Six Related Protein Products from a Single Patient with Multiple Myeloma†

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ABSTRACT: Six related myeloma protein materials were isolated from the serum and urine of a patient with multiple myeloma. These proteins were identified as an immunoglobulin G (IgG), a half-IgG, an aggregated IgG, an antigen-binding fragment (Fab), and two κ light-chain variable-region (V_L) dimers differing from each other by two arginyl residues at the carboxyl terminus. All six proteins had identical amino-terminal κ-chain sequences. Heavy-chain components had blocked amino termini and thus were not sequenced. The IgG, aggregated IgG, and half-IgG had the same amino acid compositions. The half-IgG was found to contain abnormally high quantities of carbohydrate, ~17% by weight. The monomeric and aggregated immunoglobulins had much lower levels of carbohydrate (<1%) of different composition. The patient's serum contained ~5 g/100 mL myeloma protein, of which 10% was aggregated IgG. Much smaller amounts of Fab fragment and the two V_L dimers were detectable by immunoelectrophoresis. In the urine all myeloma protein products were present in large amounts, except for the monomeric serum IgG. The high carbohydrate containing half-IgG cannot be a degradation product of the IgG since they have distinct carbohydrate compositions. Mutations affecting the carbohydrate attachment mechanisms may have occurred in one subclass of myeloma cells to account for the high carbohydrate content of the half-IgG molecules. The heavy glycosylation may have prevented the dimerization of the half-molecules to form the complete immunoglobulin. There is no direct evidence indicating whether the Fab and V_L regions are products of biosynthesis of different clones of cells derived from mutations of the original myeloma or are products of immunoglobulin degradation. In this patient, renal mechanisms may have operated to retain the monomeric IgG in the blood while concentrating the aggregated IgG of much higher molecular weight in the urine. Alternatively, the monomeric IgG may have aggregated in the kidney and the aggregated material present in the serum may represent that portion which is reabsorbed.

Multiple myeloma is a bone marrow tumor which results in the preponderance of a particular clone of antibody-producing plasma cells. Homogeneous immunoglobulins secreted by such tumors have been used for the determination of amino acid sequences, thus allowing for the development of concepts pertaining to the generation of antibody diversity and the structural complementarity of antigen and antibody, for the determination of their spatial structure by X-ray crystallography, and for biochemical studies of homogeneous antibody–antigen reactivities in those cases in which the myeloma proteins bind specifically to small chemical compounds (Nisonoff et al., 1975).

In addition to the intact myeloma protein, immunoglobulin fragments are sometimes observed in patients with multiple myeloma. Such fragments include light-chain dimers (Bence-Jones, 1848; Edelman & Gally, 1962), light chains (Deutsch, 1963), halves of light chains (Solomon et al., 1965; Cioli & Baglioni, 1966; Williams et al., 1966; Karlsson et al., 1969), heavy chains (Seligmann et al., 1968; Franklin, 1970), immunoglobulins with deletions in the heavy chain (Milstein & Frangione, 1969; Franklin & Frangione, 1971), Fab[1] fragments (Winchester et al., 1967), and half-immunoglobulins (Hobbs & Jacobs, 1969). These fragments result from either abnormalities in immunoglobulin synthesis or are merely products of degradation. For example, free light chains are probably due to a high synthetic ratio of light to heavy chains (Scharff, 1975). Those light chains not assembled into the immunoglobulin are secreted. Halves of light chains may result from the failure to link variable- and constant-region genes prior to transcription (Dreyer & Bennet, 1965; Hood & Ein, 1968; Milstein et al., 1969; Gally & Edelman, 1972). Deletions within a chain may indicate an improper gene linkage in which some of the message is not transcribed (Franklin & Frangione, 1971). That similar fragments can be isolated from cell cultures of myeloma cells is evidence for their synthetic origin (Jones, 1973; Matsuoka et al., 1969; Birshstein et al., 1974; Morrison & Scharff, 1975). On the other hand, some immunoglobulin products such as the Fc and Fab fragments appear to arise from IgG catabolism (Vaughan et al., 1967; Waldmann & Strober, 1969). Halves of light chains (V_L) could derive from enzymic cleavage in the sus-

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[1] Abbreviations used: Fab, antigen-binding fragment consisting of the light chain and the amino-terminal half of the heavy chain; Fc, "crystallizable" fragment of immunoglobulins containing disulfide-bonded carboxyl-terminal half of the heavy chain; Fd, heavy-chain component of Fab; V_L, light-chain variable region; V, variable region; C, constant region; IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; γ, α, μ, δ, and ϵ refer to the heavy chains of IgG, IgA, IgM, IgD, and IgE, respectively; κ and λ refer to the κ and λ light chains, respectively; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

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ceptible area between the V and C regions with subsequent digestion of C_L (Solomon et al., 1976; Cioli & Baglioni, 1968).

The patient studied here is unique in that his body fluids contained, in addition to the intact myeloma protein, five different proteins related to it, a remarkable diversity of such materials. Either these proteins are products of immunoglobulin catabolism or they result from abnormalities in immunoglobulin biosynthesis. In the case of a half-immunoglobulin containing 16.6% carbohydrate, a particularly high quantity possibly correlated with the appearance of *N*-acetylgalactosamine, a sugar not commonly found in human IgG myeloma proteins, it would appear likely that this material resulted from abnormalities in the biosynthesis of the immunoglobulin in a subclone of cells.

Materials and Methods

The patient studied was a 64-year-old black man diagnosed, following hospitalization, as suffering from a multiple myeloma. On admittance the patient's serum IgG level was 6.3 g/100 mL compared to normal values of 0.8–1.5 g/100 mL. IgM and IgA levels were normal. Whole-serum electrophoresis showed a monoclonal spike in the γ region. Electrophoresis of urine concentrated 50-fold showed, in addition to the proteins present in the serum, a band migrating toward the cathode. Bone marrow aspiration revealed total replacement of the marrow by immature plasma cells. The samples of urine and serum used in this study were obtained within 2 days of the start of chemotherapeutic treatment. A more detailed clinical history is given in the supplementary material (see paragraph at end of paper in regard to supplementary material).

Protein Purification. The urine was prepared for gel filtration chromatography by dialyzing large volumes against 50 mM ammonium bicarbonate and lyophilizing. Serum was dialyzed against the gel filtration buffer prior to application. Proteins in the serum and lyophilized urine (5.0-mL samples) were separated by gel filtration through a 5 × 150 cm column of Sephadex G-100 (Pharmacia) in 6.2 mM sodium borate–boric acid buffer containing 130 mM sodium chloride, pH 8.6 (Figure 2). Fractions thus obtained were dialyzed against three changes of 20 volumes of 50 mM ammonium bicarbonate for 24 h and lyophilized. Serum immunoglobulin fraction B′ (Figure 2) was fractionated on a 1.5 × 80 cm column of DEAE-cellulose (Whatman) using a linear gradient of 20–50 mM sodium phosphate, pH 8.0, total volume 500 mL (Figure 3). Fraction B′2 (2.0 mL) was subjected to gel permeation chromatography on a 2.5 × 100 cm column of Sephadex G-100 in 50 mM ammonium bicarbonate, and the two resulting fractions were lyophilized. Urine immunoglobulin fractions A and B (Figure 2) were further purified by gel filtration (2.0-mL samples) through a 2.5 × 100 cm column of Sephadex G-150 in 50 mM ammonium bicarbonate. A single run yielded pure A, while B required two gel filtrations. Urine fraction D was applied to a 1.5 × 80 cm column of DEAE-Sephadex A-50 and eluted under a linear gradient of 50–200 mM sodium chloride buffered with 6 mM sodium borate–boric acid, pH 8.6, total volume 500 mL. Further purification was on a 2.5 × 100 cm column of Sephadex G-75 (2.0-mL sample) in 50 mM ammonium bicarbonate (Figure 4). Urine fraction E was separated into two components, E1 and E2, by chromatography on a 2.5 × 60 cm column of sulfopropyl-Sephadex C-25 under a linear gradient of 0–250 mM sodium chloride in 50 mM Tris (HCl), pH 7.6, total volume 500 mL (Figure 5). In general, the purified fractions were dialyzed against 20 volumes of 50 mM ammonium bicarbonate for 48 h with several changes of the solution and lyophilized. This served to desalt those fractions which had not been gel filtered in ammonium bicarbonate and also to eliminate possible carbohydrate-containing contaminants arising from the Sephadex or the filter paper at the top of the gel column. Only immunoglobulin-related materials were purified and identified.

Characterization of Immunogobulin-Derived Materials. Purified protein fractions were identified immunoelectrophoretically (Williams, 1971) by using precast POL-E-Film (Pfizer) and specific rabbit antisera directed against human γ, μ, δ, α, ϵ, κ, and λ chains, as well as Fab and Fc fragments (Miles). The proteins were electrophoresed for 45 min at 9 V/cm and then incubated for 48 h in the presence of the antisera added to the troughs of the electrophoresis plates. The plates were washed for 48 h in 6.2 mM sodium borate buffer containing 130 mM sodium chloride, pH 8.6, the precipitin bands were stained with amido black, and the plates were dried and then destained in 5% acetic acid. Amino acid compositions were obtained from 24-, 48-, and 72-h hydrolysates of 5–10 nmol of protein in 6 N HCl at 110 °C followed by analysis on a Durrum D-500 amino acid analyzer. Amino-terminal sequences of each purified protein were obtained by Edman degradation of 100 nmol using the Beckman automatic sequencer, Model 890C, with the peptide program employing the volatile buffer dimethylallylamine (Beckman Model 890C sequencer instruction manual). Phenylthiazolinone amino acids were converted to PTH derivatives by incubation with 1 N HCl in methanol at 80 °C for 10 min. PTH amino acids were identified by gas chromatography [some residues were silylated with *N*,*O*-bis(trimethylsilyl)acetamide] (Pisano & Bronzert, 1969) employing a Beckman Model CG 45 gas chromatograph and thin-layer chromatograpy on silica gel plates (Eastman) in heptane–1,2-dichloroethane–propionic acid (11.6:5.0:3.4 v/v) (Jeppson & Sjöquist, 1967). PTH amino acids were stained with buffered ninhydrin (50 mg of ninhydrin in 50 mL of ethanol, 2 mL of collidine, and 15 mL of glacial acetic acid) and then dried at 80 °C for 20 min and identified from their chromatographic mobility and color (Nolan et al., 1973; Roseau & Pantel, 1969). In addition, PTH derivatives were hydrolyzed in hydroiodic acid for 4 h at 150 °C (Smithies et al., 1971) and the resulting amino acids identified by analyses with a Durrum D-500 amino acid analyzer. Carboxyl-terminal residues of the two proteins in fraction E were identified by amino acid analysis of the material liberated by enzymic degradation with carboxypeptidases A and B (Worthington) for different periods of time (Ambler, 1967). Thin-layer peptide maps of these two proteins on Polygram Gel 300 plates (Brinkman) were obtained following digestion with 1% trypsin by weight (Worthington) for 18 h; electrophoresis was for 45 min at 22 V/cm in pyridine–acetic acid–water (200:7:1800 v/v), and chromatography in the second dimension was in butanol–pyridine–acetic acid–water (60:40:12:48 v/v).

Molecular Weight Determinations. Estimates of molecular weights were obtained by gel filtration through Sephadex G-100 and Bio-Gel A-0.5M (Bio-Rad) (Whitaker, 1963; Andrews, 1964) and by 12 and 20% polyacrylamide gel (Eastman) electrophoresis of reduced (1% dithiothreitol) and nonreduced proteins in 1% sodium dodecyl sulfate ($NaDodSO_4$) (Weber & Osborn, 1969). Molecular weight estimates of fractions B, D, and E were also obtained by analytical ultracentrifugation (Spinco Model E ultracentrifuge) by applying the approach to equilibrium method (Chervenka, 1969). The partial specific volume ($\bar{v}$) of the protein was calculated from the amino acid composition (Cohn & Edsall,

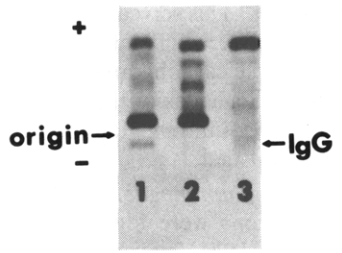

FIGURE 1: Agarose film electrophoretograms at pH 8.6 of the patient's urine (concentrated 50-fold) (lane 1), the patient's serum (lane 2), and normal human serum (lane 3). See Materials and Methods for the experimental procedure. The samples were applied at the indicated origin; the cathode is marked by – and the anode by +.

1943) and the carbohydrate content (Gibbons, 1972).

Carbohydrate Determinations. Three of the purified immunoglobulin fractions, A, B, and B′2b, were analyzed for content and types of carbohydrate. Quantification of the neutral sugars was by gas–liquid chromatography of the per-O-acetylated alditol acetates essentially by the methods of Jones & Albersheim (1972) and Meezan et al. (1976). Protein was determined by the absorbance at 280 nm, assuming $E_{1\%} = 15.0$, and hydrolyzed (200–400 μg) for 4 h at 115 °C in 2 N trifluoroacetic acid. Separation of the per-O-acetylated alditols was achieved on a 6-ft column of 4% ECNSS-M Chromosorb W (Applied Science Laboratory) at 160 °C. Known amounts of myoinositol were added as an internal standard to each sample before hydrolysis. The amino sugars were analyzed with a JOEL Model 6AH amino acid analyzer using the single-column system after hydrolysis of the proteins in 6 N HCl for 22 h at 108 °C. The monosaccharides used as standards were of the D configuration and were purchased from commercial sources.

Results

Electrophoretograms of the patient's serum and 50-fold concentrated urine and normal human serum are shown in Figure 1. Both the serum and urine show large amounts of an apparently monoclonal globulin with the urine having an additional band migrating to the cathode (Figure 1, lane 1). Since the normal serum immunoglobulin migrated farther to the cathode than the monoclonal IgG (Figure 1, lane 3), these proteins were separable by using ion-exchange chromatography as discussed below. Immunoelectrophoresis of the urine and serum samples demonstrated that the monoclonal protein was IgG containing a κ light chain, while the IgG from normal serum contained either κ or λ light chains.

The diversity of IgG-derived materials turned out to be much larger than indicated by these electrophoresis experiments, as shown by the elution profiles of the serum and urine proteins from gel filtration on Sephadex G-100 and the corresponding electrophoresis patterns shown in Figure 2. The profiles shown only involve protein materials larger than 10000 in molecular weight because of the extensive dialysis employed. Of the three fractions isolated from the serum, B′ was the only one containing proteins electrophoresing in the γ region. Fraction A′ had an unknown component of high molecular weight, and fraction C′ consisted mostly of serum albumin and several other acidic compounds. These fractions were not examined further as the immunoelectrophoresis patterns demonstrated they contained no IgG-derived proteins. Fraction B′ was separated by ion-exchange chromatography on DEAE-cellulose into two fractions (Figure 3A). Fraction B′1 was more basic than fraction B′2 since it eluted first from the anion-exchange column as expected for the normal serum immunoglobulins (see Figure 1). Furthermore, it reacted with

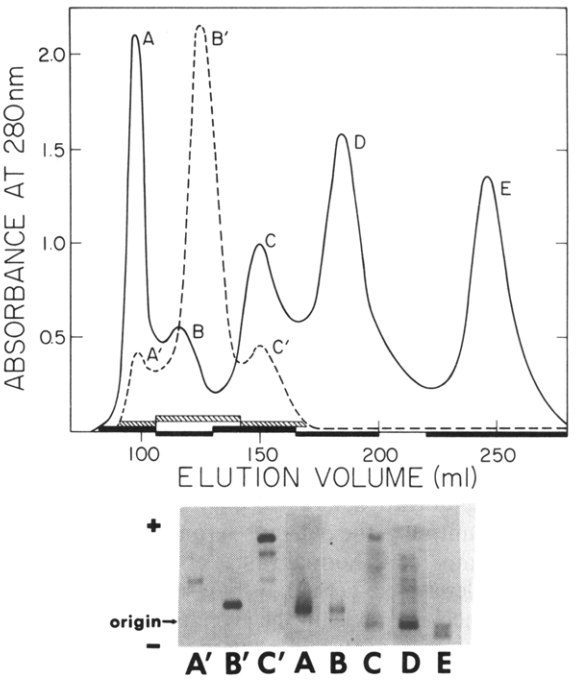

FIGURE 2: Elution profiles of the patient's urine (concentrated 100-fold; solid line) and serum (dashed line) from gel filtration on a column (5 × 150 cm) of Sephadex G-100 (top panel). The solid bars on the abscissa represent the fractions pooled from the urine chromatogram to yield fractions A–E, and the cross-hatched bars represent the fractions pooled from the serum chromatogram to yield fractions A′–C′. The bottom panel shows a drawing of agarose film electrophoretograms of these urine and serum fractions; the origin, cathode, and anode are indicated as in Figure 1.

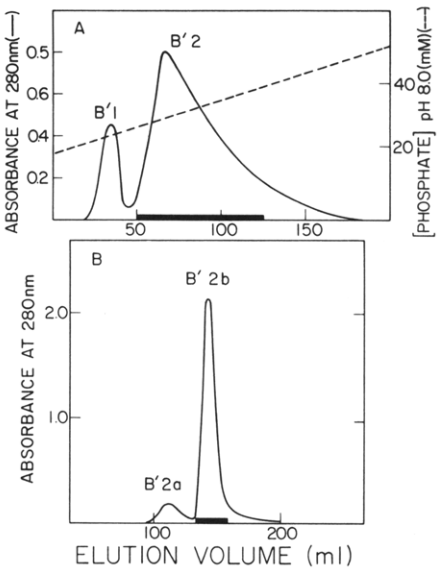

FIGURE 3: Further purification of serum fraction B′. Chromatography on DEAE-cellulose (panel A). Peak B′1 corresponded to normal serum IgG, and peak B′2 was the myeloma protein. The bar indicates the fractions pooled and subjected to gel filtration on Sephadex G-100 (panel B) to yield fractions B′2a (the aggregated myeloma IgG) and B′2b (the monomeric myeloma IgG). The procedures employed are given under Materials and Methods.

both κ and λ light-chain antisera (see Table I), indicating that it was indeed the usual heterogeneous mixture of normal serum IgG. Fraction B′2 was identical with the monoclonal serum IgG from its electrophoresis and immunological reactivity patterns. This monoclonal IgG was further purified on Sephadex G-100, separating from the monomeric protein a small amount of aggregated material (Figure 3B).

Table I: Characterization of Immunoglobulin-Derived Fractions[a]

purified myeloma products	antiserum to[b]									M_r determined by			
											electrophoresis[c]		
	μ	γ	α	δ	ϵ	κ	λ	Fc	Fab	gel filtration	original fraction	reduced protein	ultra-centrifugation
A	−	+	−	−	−	+	−	+	+	600 000	150 000	55 000 22 000	
B	−	+	−	−	−	+	−	+	+	>150 000	90 000	68 000 22 000	83 000
B'1	−	+	−	−	−	+	+	+	+	150 000			
B'2b	−	+	−	−	−	+	−	+	+	150 000	150 000	55 000 22 000	
D	−	+	−	−	−	+	−	−	+	43 000	41 000	23 000 18 000	34 000
E1	−	−	−	−	−	+	−	−	−	17 800	18 000	9 000	17 000
E2	−	−	−			+	−	−	−	17 800	18 000	9 000	

[a] The six myeloma products were purified as given in the text and tested against the variety of antisera listed, and their molecular weights were determined by gel filtration, by $NaDodSO_4$–polyacrylamide gel electrophoresis before and after reduction with 1% dithiothreitol, and by ultracentrifugation. For comparison, fraction B'1, which is normal IgG obtained from the patient, is also included. [b] The occurrence of a specific precipitation reaction between the antiserum and the purified fraction (+) or the lack of precipitation (−) was determined by immunoelectrophoresis. [c] By $NaDodSO_4$–polyacrylamide gel electrophoresis.

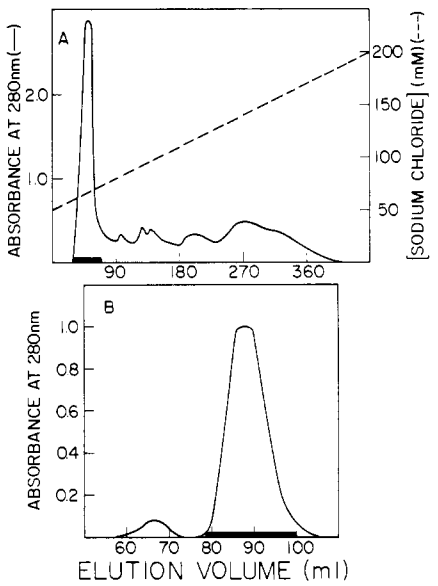

FIGURE 4: Further purification of urine fraction D. Chromatography on DEAE-Sephadex A-50 (panel A). The bar indicates the fractions pooled and subjected to gel filtration on Sephadex G-75 (panel B). The final purified fraction D (myeloma Fab) was obtained from the fraction pooled as indicated. The procedures employed are given under Materials and Methods.

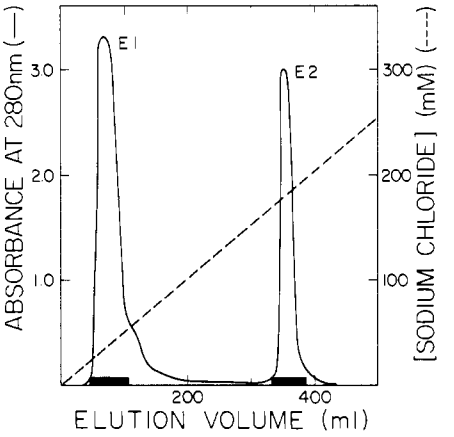

FIGURE 5: Further purification of urine fraction E by column chromatography on sulfopropyl-Sephadex C-25, as given under Materials and Methods. E1 and E2 are the two light-chain variable-region proteins which separate by cation-exchange chromatography as expected from their electrophoretic mobilities (see Figure 2, bottom panel).

Urine fractions A and B had the same electrophoretic mobility (Figure 2), and they were purified by gel filtration on Sephadex G-150. Fraction C consisted mostly of serum albumin as determined by its electrophoretic mobility (Figure 2) and the fact that it did not react with any of the anti-immunoglobulin sera. Urine fraction D was chromatographed on DEAE-Sephadex followed by gel filtration on Sephadex G-75 (Figure 4). The final main fraction was judged to be pure from its behavior on $NaDodSO_4$–polyacrylamide gel electrophoresis and the fact that the ultracentrifugation experiment yielded a linear plot of the logarithm of the protein concentration as a function of the distance of the boundary from the meniscus. Two proteins were isolated from urine fraction E by using cation-exchange chromatography on sulfopropyl-Sephadex C-25 (Figure 5).

The immunologic reactivities of the purified protein fractions are listed in Table I along with their molecular weights as determined by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$, by ultracentrifugation, and by gel filtration. Fractions A, B, and B'2 reacted with antisera against γ and κ chains as well as against Fab and Fc fragments. They did not react with antisera against μ, α, δ, ϵ, and λ chains. On this basis, it was concluded that these three materials probably contained completely synthesized κ light and γ heavy chains. Fraction D also precipitated with the same sera but did not react with anti-Fc sera and therefore most probably lacked the Fc domains. Fractions E1 and E2 reacted only with anti-κ-chain antisera and thus presumably lacked any heavy-chain components.

Purified fraction A had an approximate molecular weight of 600 000. However, since the protein did not elute at a volume corresponding to the linear region of the molecular weight plot for the calibrated Bio-Gel A-0.5M column, it is not certain whether this aggregated immunoglobulin consisted of four or more IgG monomers. Fractions B'2b and B'1 both had molecular weights of 150 000, as expected for IgG. Fraction B migrated much faster on Sephadex G-100 (Figure 2) than expected from the molecular weight estimate of 83 000 obtained by ultracentrifugation. Furthermore, the results of $NaDodSO_4$–polyacrylamide gel electrophoresis indicated a molecular weight of 90 000 for this fraction. Thus, it appears

Table II: Amino-Terminal Sequences of Immunoglobulin-Derived Fractions[a]

5 10 15
Glu-Ile-Val-Met-Thr-Gln-Ser-Pro-Ala-Thr-Leu-Ser-Leu-Ser-Pro-Gly-
20 25
Glu-Arg-Ala-Thr-Leu-Ser-Cys-Thr-Ala

[a] The amino acid sequence listed was obtained by automatic Edman sequential degradation for all six purified myeloma products obtained from the serum and the urine of the patient (see text). The cysteine at position 23 was assumed from homology with κ light-chain sequences. The other residues were determined experimentally.

Table III: Amino Acid Compositions of Purified Myeloma Products[a]

	molecules of amino acid/molecule of protein					
	A	B'2b	B	D	E1	E2
Asp	115	110	123	28	10	10
Thr	117	113	114	32	16	16
Ser	141	150	135	40	17	17
Glu	152	154	152	42	17	17
Gly	91	94	97	21	14	14
Ala	88	85	88	31	18	18
Val	115	117	106	26	8	8
Met	15	15	18	2	1	1
Ile	41	37	43	11	8	8
Leu	123	118	116	28	13	13
Tyr	43	40	44	14	6	6
Phe	47	41	42	18	9	9
His	26	30	30	3	1	1
Lys	74	81	75	19	5	5
Arg	46	44	47	13	6	8

[a] The amino acid compositions of the myeloma product fractions refer to averages obtained from duplicate hydrolysates at 24, 48, and 72 h in 6 N HCl at 110 °C except for serine and threonine, the values for which were extrapolated to 0 time. The numbers were rounded off to the nearest integer. Proline, cysteine, and tryptophan were not determined.

that in its native conformation fraction B had a much larger Stokes' radius than expected of globular proteins of that molecular weight as may result from its content of near to 17% by weight carbohydrate, discussed below. No such discrepancies were observed with fractions D, E1, and E2, the molecular weights determined by all three procedures giving comparable results (Table I).

Each of four of the proteins could be separated into two polypeptides when reduced and electrophoresed in 20% polyacrylamide gels in the presence of 1% $NaDodSO_4$ (see Table I). Fractions A, B, and B'2b consisted of both light and heavy chains. In addition, much smaller amounts of materials shorter than light chains were observed on $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions. These materials could not be further characterized. These bands may represent incompletely synthesized light chains incorporated into the immunoglobulin or products of secondary degradation. Since they appeared to be attached by disulfide bonds to the heavy chains, if these were indeed products of aberrant biosynthesis they were different from the V_L fragments of fractions E1 and E2 (see below). Fraction D, when reduced, separated into two bands of approximate molecular weights 23 000 and 18 000. Fractions E1 and E2 migrated with an apparent molecular weight of 18 000, which on reduction became 9 000, indicating the presence of disulfide-bonded dimers in both of these materials.

On the basis of their immunologic reactivities, molecular weights, and subunit compositions, the six protein materials isolated from the serum and urine are thus identified as follows: (A) aggregated IgG; (B) half-IgG; (B'2b) IgG; (D) Fab fragment; and (E1 and E2) V_L dimers.

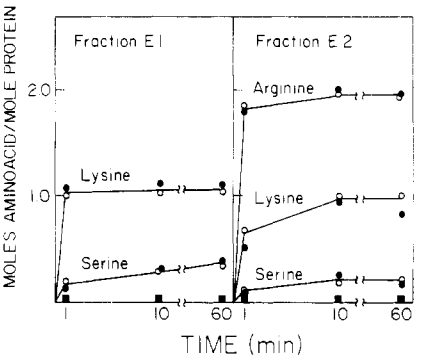

FIGURE 6: Carboxypeptidase digestion of fractions E1 and E2. The proteins (100 nmol) in 5.0 mL of 0.25 M *N*-ethylmorpholine acetate, pH 8.5, were incubated at 37 °C with 10% (w/w) carboxypeptidase A (■), 10% (w/w) carboxypeptidase B (○), or a mixture of both (●). Aliquots of 1.0 mL were removed at intervals and put in a boiling water bath for 3 min, the precipitated protein was centrifuged off, the supernatant was dried by evacuation, the residue was dissolved in the amino acid analyzer buffer, and the amino acid content was determined on a Durrum 500 amino acid analyzer. The only residues liberated were those noted in the figure.

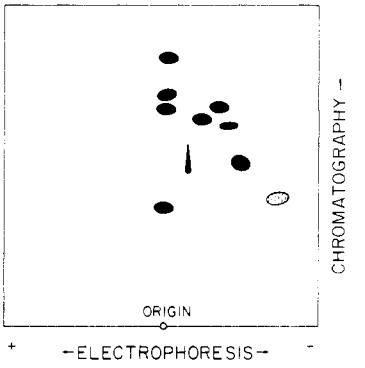

FIGURE 7: Drawing of thin-layer peptide maps of tryptic digests of fractions E1 and E2. The origin of the electrophoresis is indicated by the open circle; + indicates the anode and – the cathode. The two fractions gave identical peptide maps, except for the cross-hatched spot which was present only in the digest of fraction E2 and had a mobility indistinguishable from that of arginine.

The six purified proteins were all found to possess the same amino-terminal amino acid sequence (Table II). This sequence is clearly that of a κ III light-chain subclass protein, with the exception of a threonyl residue instead of arginine at position 24 (Kabat et al., 1976). This was the only sequence obtained from those proteins which also contained heavy chains, indicating that the heavy chains had a blocked amino terminus.

The amino acid compositions of the purified myeloma protein materials are given in Table III. The IgG (B'2b), aggregated IgG (A), and half-IgG (B) have the same amino acid contents. The two V_L dimers, E1 and E2, are identical, except for the two extra arginyl residues which have been shown to be at the carboxyl terminus of E2 by carboxypeptidase degradation (Figure 6). Indeed, while only a single lysyl residue was liberated by carboxypeptidase B or a mixture of carboxypeptidases B and A from fraction E1, the same digestion for fraction E2 contained in addition two residues of arginine, which appeared to be liberated somewhat earlier than the lysine. In both cases a small amount of serine appeared, and no other amino acids whatsoever. Carboxypeptidase A produced no free amino acids from either protein. Although fractions E1 and E2 would appear to be dimers, only one lysine was observed for each at or near the carboxyl terminus. This may indicate some asymmetry between the

Table IV: Carbohydrate Compositions of Immunoglobulin Fractions[a]

	B		A		B'2b	
fucose	0.9	4.5	ND[b]		ND	
mannose	2.8	13.8	0.07	0.95	0.23	2.7
galactose	6.5	32.7	0.03	0.36	0.03	0.36
GlcNAc	3.2	16	0.23	2.8	0.06	0.71
GalNAc	1.5	7.5	ND		0.01	0.12

[a] The first column under each fraction represents the weight percent and the second column refers to the moles of carbohydrate per mole of protein. The molecular weights used to calculate the moles of protein were 90 000 for fraction B and 150 000 for fractions A and B'2b. These are the half-immunoglobulin, the aggregated IgG, and the monomeric IgG, respectively. [b] None detected.

two chains in each dimer. The two arginyl residues at the carboxyl terminus of E2 are clearly visible as the additional ninhydrin-positive spot in the comparison of the tryptic peptide maps of E1 and E2 (Figure 7), which was identified as free arginine. There are a total of nine distinct peptides in the peptide map of fraction E1 (Figure 7). While there are five lysyl and six arginyl residues in the amino acid composition of this protein (Table III), one of the lysines is carboxyl-terminal so that one may expect a total of ten tryptic peptides. The presence of only nine such peptides probably indicates that tryptic digestion at one of the basic residues is hindered.

The carbohydrate compositions of the IgG (B'2b), aggregated IgG (A), and half-IgG (B) are given in Table IV. Of the three, only the half-IgG (B) contained substantial amounts of carbohydrate, including GalNAc. The other IgG fractions contained only small amounts of galactose, mannose, and GlcNAc and no detectable fucose or GalNAc. The total content of carbohydrate for the half-IgG (B) was 16.6%, and the molar ratio fucose/mannose/galactose/GlcNAc/GalNAc was 1:3.1:7.3:3.6:1.7. The calculated contribution of the carbohydrate moieties to the molecular weight is ~13 000. If the latter number is added to the molecular weight (75 000) of a half-IgG, the total becomes 88 000. This number compares favorably with the 83 000 value estimated by ultracentrifugation and the 90 000 value estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis. The carbohydrate contents of the other two fractions are low, perhaps indicating that only a fraction of such molecules are glycosylated.

Discussion

By taking into account the number of subunits, the molecular weight, and the immunologic specificities, we identified the six myeloma protein products as immunoglobulin G, aggregated IgG, half-IgG, Fab, and two V_L dimers differing from one another by only two arginyl residues at the carboxyl terminus. All of these proteins are related since they all have the same amino-terminal κ-chain sequence. In addition to these, the patient's urine and serum contained much smaller amounts of other myeloma protein products, including Fab fragments and V_L region peptides in the serum as well as an Fc fragment in the urine, but these could only be detected by immunoelectrophoresis and were not isolated. Since no information concerning the structures of these fragments could be obtained, it is not possible to consider whether or not they represent proteolytic digestion products rather than the results of biosynthetic abnormalities.

The most remarkable of the myeloma materials is the half-IgG that contains 16.6% carbohydrate by weight, giving the molecule a molecular weight of around 90 000. Since protein glycosylation is known to occur intracellularly (Schachter et al., 1970), the half-IgG is not a fragment of the serum myeloma protein resulting from processing after secretion. Furthermore, it is highly unlikely that the heavily glycosylated half-IgG is itself processed extracellularly into the monoclonal IgG of usual carbohydrate content. Normally, IgG is secreted from cells in the completely dimerized state. Thus, it would appear that the present half-IgG results from synthesis by a subclone of myeloma cells in which a mutation has occurred allowing greater glycosylation. The large amount of carbohydrate may be due either to longer oligosaccharide chains or to more of them. IgG myeloma proteins contain an oligosaccharide chain in the Fc portion, and approximately one-third of those examined also have one or more oligosaccharides in the Fab section distributed at three possible attachment sites on the light chains and at one site on the Fd segment (Spiegelberg et al., 1970). Normal light chains are rarely if ever glycosylated. The normal mannose/GlcNAc/galactose molar ratios of constituent sugars of human IgG are 3:4–6:1–2, with one GlcNAc binding the oligosaccharide to an asparagine on the polypeptide chain and the remainder within the oligosaccharide (Kornfeld et al., 1971). Because mannose is found only on oligosaccharide chains linked to the protein through GlcNAc, the mannose content of 13.8 residues/molecule of half-IgG would thus correspond to an average of 4.6 oligosaccharide chains/half-molecule, each of which may contain 4–6 residues of galactose, for a total of 18–28 galactose residues. The remaining galactose residues may then be in oligosaccharide chains with GalNAc at the terminal. Many other possible oligosaccharide compositions would be compatible with the data, and the number of chains linked to the protein through GlcNAc may well be greater than five. Not enough purified half-IgG was obtained from the urine to attempt to identify the number and positions of oligosaccharide chains per half-molecule.

Some indication of the nature of the abnormality may be afforded by the presence of GalNAc in these oligosaccharide chains, a sugar which is not normally observed in human IgG but which has been found in the material from a patient with heavy-chain disease (Clamp et al., 1968) and which does occur in rabbit IgG (Fanger & Smyth, 1972) and human IgG (Grey et al., 1971; Dawson & Clamp, 1968) myeloma proteins. In those cases, GalNAc is linked directly to the polypeptide chain to serine and/or threonine. It would appear that in the present case the GalNAc transferase is operative. The carbohydrate transferase systems may be particularly activated in the subclone, producing the glycosylated half-IgG. Alternatively, the conformation of the assembled half-IgG may have been altered dramatically by other mutations or deletions in the protein sequence, allowing extensive glycosylation. The latter situation seems unlikely since the amino acid composition of the half-IgG appears to be the same as the monomeric, low carbohydrate containing IgG. It may well be that the presence of the large amount of carbohydrate by itself was sufficient to prevent the dimerization of the half-molecules to the normal IgG structure rather than the occurrence of a second mutational event within the cells causing a specific abnormality in the dimerization process.

The other products which may result from biosynthetic abnormalities are the two light-chain V_L regions, since in those cases in which light-chain fragments are observed as a result of extracellular proteolysis there is an even larger amount of intact light chains (Solomon & McLaughlin, 1969). In the present case no free light chains or light-chain dimers could be detected. Furthermore, proteolytic products are likely to be much more heterogeneous than the two variable regions

isolated and there was no indication that any other similar products were present in amounts of more than traces of those of the isolated materials. However, it is not clear whether or not one of the isolated V_L regions results from proteolytic removal of the arginyl residues at the carboxyl terminus of the other.

If the abnormality leading to the excretion of these variable regions is biosynthetic, then it would appear to result from (1) an error in the mechanism by which the separate gene segments which code for an immunoglobulin chain are brought together (Tonegawa et al., 1977), (2) discontinuity of mRNA transcription beyond the gene segment coding for the V_L region, or (3) incorrect processing of the precursor message containing the intervening sequence so as to eliminate all but the segment corresponding to the variable region. In this connection it is interesting to note that the amino acid residue corresponding to the last codon amino-terminal to a large intervening sequence in the DNA coding for the B chain of mouse hemoglobulin is arginine (Tilghman et al., 1978), the same residue found at the carboxyl terminus of the variable-region peptides in the present case as is common for light chains in general. Whether the arginines located at the amino and carboxyl termini of the connecting peptides of proinsulins (Tager & Steiner, 1974) represent a similar phenomenon or are merely required for the proteolytic processing of the hormone is unclear.

The present data do not permit a decision as to the origin of the Fab fragment isolated from the patient's urine. The fact that a trace of Fc fragment could also be observed would seem to indicate that the Fab was the result of proteolytic degradation of the whole IgG, the Fc fragment being largely proteolyzed. On the other hand, it is possible that the Fab fragment is a product of abnormal synthesis of the heavy chain in which only the V_H and C_{H1} domains are made while the very small amount of Fc results from proteolysis of whole IgG.

The urine of the patient contained quite different proportions of the myeloma protein products than did the serum. Thus, as compared to serum albumin as an internal standard (1.0 mol), the serum contained 1.3 mol of monomeric IgG per mol of albumin, while in the urine there were 0.44 mol of aggregated IgG, 0.92 mol of half-IgG, no monomeric IgG, 1.4 mol of Fab, and 2.2 mol of light-chain variable-region peptides per mol of albumin. This was calculated from the results shown in Figure 2, assuming $E_{1\%} = 6.0$ for albumin and $E_{1\%} = 15.0$ for immunoglobulin-related materials.

The monomeric IgG was not excreted even though a higher molecular weight aggregated IgG was apparently preferentially cleared into the urine. Even though the polypeptide chains are apparently the same for these two myeloma products, it may well be that their differences in oligosaccharide chains are responsible for clearing one from the blood and retaining the other. Similarly, the recognition of carbohydrate moieties by receptors has been proposed by Baynes & Wold (1976) as being involved in the clearance of antibody–antigen complexes. Whether the differential excretion in the present case does or does not involve renal readsorption has not been examined.

The excretion into the urine of Fab fragments and light chains or Bence-Jones proteins is a well-known phenomena (Waldmann & Strober, 1969), and it can be presumed that in the present case the highly glycosylated half-IgG and the light-chain fragments concentrated in the urine behave in a similar fashion.

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Supplementary Material Available

The clinical history of the patient is described (3 pages). Ordering information is given on any current masthead page.

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Affinity Labeling of a Lysine Residue in the Coenzyme Binding Site of Pig Heart Mitochondrial Malate Dehydrogenase†

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ABSTRACT: Porcine heart mitochondrial malate dehydrogenase is inactivated with a pseudo-first-order rate constant of 0.00941 min^{-1} by 2.1 mM 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine in 0.02 M sodium barbital buffer, pH 8.0, containing 15% dimethylformamide (DMF). The pseudo-first-order rate constant for inactivation is linearly dependent on the reagent concentration. The adenosine moiety is crucial, as indicated by the observation that *p*-(fluorosulfonyl)benzoic acid (which lacks the adenosine group) inactivates the enzyme 37-fold more slowly. Marked protection against this inactivation is provided by NADH and NAD, but not by malate. The dissociation constants for NADH and NAD, calculated from the decrease in the inactivation rate, are 4.6 and 385 μM, respectively, values close to those obtained by previous direct binding measurements. This dimeric enzyme is known to be dissociated to its monomeric form at low protein concentration and low pH. However, sedimentation equilibrium and light-scattering studies reveal that the native enzyme retains its dimeric structure in the presence of 15% DMF under the conditions of reaction with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, and gel filtration experiments demonstrate that the state of aggregation of the enzyme is not altered upon modification by this reagent. A plot of the incorporation of [^{3}H]-5′-(*p*-sulfonylbenzoyl)adenosine vs. loss of enzyme activity is linear throughout the range tested, and extrapolation leads to 0.97 mol of radioactive reagent incorporated per mol of enzyme subunit at 100% inactivation. Fractionation of the acid hydrolysate of the modified enzyme by amino acid analysis or by two-dimensional thin-layer chromatography and electrophoresis leads to the identification of lysine as the modified amino acid. These results indicate the presence of a lysine residue at or near the coenzyme binding site of porcine heart mitochondrial malate dehydrogenase.

Pig heart mitochondrial malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) is a dimeric enzyme which uses NAD as a cofactor. Although histidine, arginine, and lysine residues have been implicated as being in the catalytic site on the basis of chemical modification with general protein reagents (Anderton, 1970; Anderton & Rabin, 1970; Gregory et al., 1971; Foster & Harrison, 1974; Wimmer & Harrison, 1975), the coenzyme binding site has yet to be fully mapped. Affinity labeling has not thus far been applied in the case of malate dehydrogenase to limit the extent of chemical modification to the region of the active site. We have previously described the synthesis of 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine, which has been used to label stoichiometrically the NADH regulatory site of glutamate dehydrogenase (Pal et al., 1975) and the adenosine nucleotide sites of rabbit muscle pyruvate kinase (Wyatt & Colman, 1977), RNA polymerase (Kumar et al., 1977), phosphofructokinase (Mansour & Colman, 1978; Pettigrew & Frieden, 1978), mitochondrial F_1 ATPase (Esch & Allison, 1978), chloroplast ATPase (DeBenedetti & Jagendorf, 1979), and human platelet membranes (Bennett et al., 1978). 5′-[*p*-

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