The C-Terminal Sequences of the Heavy Chains of Human Immunoglobulin G Myeloma Proteins of Differing Isotypes and Allotypes

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The sequences of the C-terminal octadecapeptides obtained by cyanogen bromide cleavage of the γ -chains of myeloma proteins of the four subclasses, and a urinary heavy-chain-disease protein, have been determined. Although the sequences were markedly homologous, unique replacements were identified that distinguished between the γ_{2b} , γ_{2c} and γ_{2d} subclasses. The data are in accord with the postulated existence of four genetic loci or cistrons, these having arisen by the process of gene duplication.

The nomenclature employed here for human immunoglobulin G (IgG), its fragments and its genetic factors, is that recommended by the World Health Organisation (1964, 1965).

Two kinds of antigenic specificities (allotypic and isotypic) of different genetic significance have been recognized in immunoglobulins. Isotypic specificities are those present in all normal individuals of the same species; for example, it is these specificities that make it possible to distinguish between the main classes of immunoglobulins, IgG, IgA and IgM. Further, such specificities are involved in the recognition of the four subclasses of human IgG by primate and rabbit antisera against normal human immune IgG and myeloma proteins, the determinants being restricted to the γ -chains (Grey & Kunkel, 1964; Ballieux, Bernier, Tominaga & Putnam, 1964; Terry & Fahey, 1964). These four antigenically distinct subclasses of γ -chain are known as γ_{2a} , γ_{2b} , γ_{2c} and γ_{2d} , or Ne, We, Vi and Ge respectively.

Conversely, allotypic specificities are those which differ among individuals or groups of individuals within the same species. This genetic polymorphism of human immunoglobulin was first appreciated by Grubb (1956) and Grubb & Laurell (1956) with the elucidation of Gm(1), and has now been extended to include more than twenty Gm factors or specificities. The Gm specificities appear to be associated with the γ -chains of IgG, but not with the α - or μ -chains of IgA or IgM (Fahey & Lawler, 1961; Mårtensson, 1962; Harboe, Osterland & Kunkel, 1962; Lawler & Cohen, 1965). Extensive population studies have been undertaken in an attempt to define the genetics of the Gm specificities (Steinberg, 1962; Mårtensson, 1966), but it has been through the use of

myeloma proteins that an understanding of the linked relationship between the subclasses and the inheritance of the Gm specificities has evolved (Kunkel, Allen, Grey, Mårtensson & Grubb, 1964; Terry, Fahey & Steinberg, 1965).

Recently the structural variations responsible for the antigenic specificities determining subclass and Gm factor have been investigated by peptide 'fingerprinting' of the tryptic digests of the Fc fragments of myeloma proteins of known isotype and allotype (Thorpe & Deutsch, 1966; Frangione, Franklin, Fudenberg & Koshland, 1966). A distinctive peptide was found to be associated only with the γ_{2b} :Gm(1) myeloma proteins, but unfortunately this peptide did not appear to possess allotypic antigenic determinants. Similar 'fingerprints' were obtained for proteins of the same subclass, yet significant differences were demonstrated between the subclasses, particularly between γ_{2b} , γ_{2c} and γ_{2d} . In their structural studies of the γ -chain of a pathological human IgG immunoglobulin of subclass and Gm type γ_{2b} :Gm(1), Press, Piggot & Porter (1966) have reported the sequence of the C-terminal octadecapeptide obtained by cyanogen bromide cleavage. An identical peptide has been recovered in good yield from normal IgG (Piggot & Press, 1966). However, examination of a heavy-chain-disease protein and a myeloma protein of type γ_{2c} :Gm(5, 13, 14) revealed that amino acid replacements can occur in the C-terminal octadecapeptide (Prah, 1966). The investigation of this C-terminal sequence has been extended to myeloma proteins of other subclasses and phenotypes.

MATERIALS

The myeloma proteins of Mar, Wan and She were supplied as sera and the urinary heavy-chain-disease protein, Zuc, as an $(\text{NH}_4)_2\text{SO}_4$ paste by Professor E. F. Osseman of the

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Table 1. *Subclass and Gm specificities of the myeloma proteins*

The two alternative nomenclatures for subclass and for Gm specificity are shown (World Health Organisation, 1964, 1965).

Myeloma protein	Subclass	Gm specificity					
		1 (a)	4 (f)	5 (b ¹)	13 (b ³)	14 (b ⁴)	21* (g)
Wan†	γ_{2a} (Ne)	—	—	—	—	—	—
Daw	γ_{2b} (We)	+	—	—	+	—	—
Zuc	γ_{2c} (Vi)	—	—	+	+	+	—
Mar	γ_{2c} (Vi)	—	—	+	+	+	—
Vil	γ_{2c} (Vi)	—	—	—	—	—	+
She‡	γ_{2d} (Ge)	—	—	—	—	—	—
Ger‡	γ_{2d} (Ge)	—	—	—	—	—	—

* Natvig (1966).

† Gm(n) specificity unknown (Kunkel, Yount & Litwin, 1966).

‡ No Gm specificity has been identified in the γ_{2d} (Ge) subclass.

College of Physicians and Surgeons, Columbia University, New York, N.Y., U.S.A., and the sera of Vil and Ger by Professor H. G. Kunkel of the Rockefeller University, New York, N.Y., U.S.A. The isotypic and allotypic information available for these proteins is shown in Table 1.

Human myeloma proteins. These were isolated from the sera by Na_2SO_4 precipitation (Kekwick, 1940) followed by chromatography on DEAE-Sephadex A-50. The precipitate obtained from 10 ml. of serum at 18% (w/v) Na_2SO_4 was redissolved and reprecipitated at 14% (w/v) Na_2SO_4 . This precipitate was dissolved in 10 ml. of 0.15 M-NaCl, dialysed exhaustively against 0.02 M-potassium phosphate buffer, pH 6.3, in the cold and applied to a DEAE-Sephadex A-50 column (1.6 cm. $\times$ 30 cm.); after the column had been eluted with 100 ml. of the same buffer, a linear gradient of 0.02–0.20 M-potassium phosphate buffer (300 ml. of each), pH 6.3, was developed. The extinction of the eluate was measured at 280 m μ ; appropriate fractions were pooled, concentrated by pressure dialysis against 0.15 M-NaCl and freeze-dried. Each fraction was subjected to electrophoresis on cellulose acetate, and only those fractions that gave a single sharp band were employed for further studies.

Urinary heavy-chain-disease protein, Zuc. The $(\text{NH}_4)_2\text{SO}_4$ paste of the urinary proteins was dissolved in the minimum volume of water and dialysed against 0.05 M- NH_4HCO_3 in the cold with frequent changes of the NH_4HCO_3 solution. The dialysed protein was then fractionated on Sephadex G-75 in the same buffer, from which it was eluted at 35–50% of the total column volume; this fraction was pooled and concentrated. Contamination with pigmented protein, presumably transferrin, was still evident. Approximately 500 mg. of the freeze-dried protein was dissolved in 10 ml. of 0.02 M-potassium phosphate buffer, pH 6.3, dialysed against the same buffer, and chromatographed on DEAE-Sephadex A-50 as described above for the serum myeloma proteins. More than 90% of the applied protein was eluted in a single peak between 0.09 M- and 0.12 M-phosphate buffer, pH 6.3, and gave a single sharp band when subjected to electrophoresis on cellulose acetate.

Enzymes. Trypsin, chymotrypsin and carboxypeptidase A were purchased from Worthington Biochemicals Corp., Freehold, N.J., U.S.A. The chymotryptic activity of the trypsin was reduced by treatment with chloromethyl

L-(2-phenyl-1-toluene-*p*-sulphonamido)ethyl ketone by the method of Kostka & Carpenter (1964). Carboxypeptidase A was treated with di-isopropyl phosphorofluoridate as described by Potts, Young, Anfinsen & Sandoval (1964).

Dithiothreitol (Cleland's reagent). This was purchased from Calbiochem, Los Angeles, Calif., U.S.A.

Anhydrous hydrazine. This was prepared from hydrazine hydrate (Kusama, 1957).

METHODS

Reduction and isolation of chains. Initially the myeloma proteins (20 mg./ml.) were reduced with 2-mercaptoethanol at a concentration of 0.10 M (approx. 130-fold molar excess relative to myeloma protein) at room temperature (Fleischman, Pain & Porter, 1962). Alkylation was performed at 0° by the addition of recrystallized iodoacetamide to a final concentration of 0.11 M, and the reaction mixtures were dialysed against cold 0.15 M-NaCl overnight and then for a further 24 hr. against cold *n*-propionic acid. In later experiments partial reduction was effected by 5 mM-dithiothreitol (Cleland, 1964; approx. sevenfold molar excess relative to myeloma protein) in 0.50 M-tris-HCl, pH 8.2, containing 2 mM-EDTA, at 37° for 2 hr., subsequent alkylation with iodoacetamide (10 μ M) being carried out at 0°.

Separation of the chains of the partially reduced proteins was accomplished by fractionation on Sephadex G-75 columns in *n*-propionic acid at room temperature. The γ -chain was concentrated by pressure dialysis against 0.1 N-acetic acid and freeze-dried. On a weight basis the recovery of γ -chain and light chain varied with individual myeloma proteins from 60% to 68% and from 26% to 32% respectively.

Total reduction of the γ -chain was carried out in 7 M-guanidine-HCl–0.5 M-tris-HCl, pH 8.2, at a dithiothreitol concentration of 0.1 M for 2 hr. at 37°. After alkylation in the cold, the reagents were removed by dialysis against 0.5 N-acetic acid.

Cyanogen bromide cleavage of γ -chains. This was carried out in 70% (v/v) formic acid as described by Press *et al.* (1966), except that the reaction mixture was kept at room temperature, rather than 25°, for 24 hr.

Amino acid analysis. Conditions similar to those described by Crumpton & Wilkinson (1963) were followed to determine the amino acid composition of the γ -chains, with a Spinco amino acid analyser, model 120, equipped with 55 cm. and 8 cm. columns for the separation of acidic and neutral amino acids, and basic amino acids respectively. The half-cystine and methionine contents were determined on performic acid-oxidized samples (Moore, 1963) as cysteic acid and methionine sulphone respectively on the 150 cm. column of the amino acid analyser. Tryptophan was estimated spectrophotometrically (Bencze & Schmid, 1957).

Peptide hydrolysates were analysed on a similar instrument modified for accelerated analysis (Benson & Patterson, 1965) and equipped with a flow cell of 10 cm. light-path (Evans Electroselenium Ltd., Halstead, Essex); satisfactory analysis were obtained with 0.01 μ mole of peptide.

Before analysis for homoserine, the hydrolysate was dissolved in 1 ml. of pyridine-acetate buffer, pH 6.5, and boiled for 1 hr. to convert homoserine lactone into homoserine (Ambler, 1965). The pH of the buffer used to elute homoserine from the 55 cm. column of the analyser was adjusted from 3.25 to 3.15–3.20 with conc. HCl.

Hydrazinolysis. This was carried out according to the method of Bradbury (1958) with hydrazine sulphate as catalyst.

Other methods. The solvents and method of high-voltage paper electrophoresis have been described by Crumpton & Wilkinson (1965); mobilities are expressed relative to a lysine marker. Peptides were stained with ninhydrin or for histidine, arginine and tryptophan as outlined by Easley (1965). The techniques of cellulose acetate and starch-gel electrophoresis have been described in detail by Press *et al.* (1966). Enzymic digests were performed in 0.1 M-NH₄HCO₃ for 2 hr. at 37° at an enzyme concentration of 0.20 mg./ μ mole of peptide. Subtractive Edman degradation of peptides was carried out according to Konigsberg & Hill (1962); the composition of the resultant peptide and its percentage recovery are reported.

RESULTS

Preparation and properties of myeloma proteins

The purification of the myeloma proteins by chromatography on DEAE-Sephadex was markedly influenced both by the ratio of myeloma protein to residual normal IgG and by the subclass of the myeloma protein being purified. In those sera containing appreciable amounts of normal IgG (> 30–40% of the total IgG), it was necessary to divide the eluted protein into multiple small fractions, only those fractions that gave a sharp single band on cellulose acetate electrophoresis being selected. As anticipated, a correlation between behaviour on cellulose acetate electrophoresis and that on chromatography on DEAE-Sephadex was observed; those myeloma proteins that migrated in the γ_1 – γ_2 region (i.e. γ_{2a} , γ_{2b} and γ_{2c}) were unretarded or only weakly retarded in the 0.02 M-phosphate buffer, pH 6.3. In contrast, the myeloma proteins of the γ_{2d} subclass were strongly retarded, elution not occurring until the phosphate

gradient had reached 0.08–0.10 M. On cellulose acetate electrophoresis these proteins migrated in the β_2 – γ_1 region. Although the urinary heavy-chain-disease protein was also strongly retarded on DEAE-Sephadex chromatography, on cellulose acetate electrophoresis it migrated in the γ_1 – γ_2 region. No fractionation of this protein into components of low and high sialic acid content was observed on chromatography on DEAE-Sephadex, as has been reported by Spiegelberg & Weigle (1966) on DEAE-cellulose.

After alkylation of the partially reduced myeloma proteins, excess of iodoacetamide was removed by dialysis against 0.15 M-sodium chloride to prevent alkylation of the methionine residues (Gundlach, Stein & Moore, 1959) when the pH was subsequently decreased for the dissociation and Sephadex fractionation of the chains. At this stage several of the myeloma proteins showed a tendency to gel when 2-mercaptoethanol had been used for reduction; this was circumvented by the substitution of dithiothreitol (Cleland, 1964). Starch-gel electrophoresis in 8 M-urea-formate, pH 3.7, of the γ -chains revealed no obvious contamination by light chain. The amino acid compositions of the γ -chains are shown in Table 2. A marked increase was observed in the susceptibility of the disulphide bonds of myeloma proteins to reduction with 2-mercaptoethanol compared with those of normal IgG (Crumpton & Wilkinson, 1963), which may in part account for the gelling phenomenon. This is evident from the *S*-carboxymethylcysteine content of the γ -chains shown in Table 2, where all had been isolated from myeloma proteins reduced with 0.1 M-2-mercaptoethanol, with the exception of the Ger protein, which had been reduced with 5 mM-dithiothreitol. The *S*-carboxymethylcysteine content of the Vil γ -chain decreased from 10 to 4.6 moles/50 000 g. if dithiothreitol was used for the reduction. Glycine was established as the *C*-terminal residue in each of the γ -chains investigated and in the heavy-chain-disease protein, Zuc, hydrazinolysis yielding 0.55–0.70 mole of glycine (uncorr.)/50 000 g. of γ -chain protein and 0.64 mole of glycine (uncorr.)/26 000 g. of the heavy-chain-disease protein. Initial attempts to degrade the γ -chains of the intact myeloma proteins or partially or totally reduced γ -chain in the presence of 2 M-urea by carboxypeptidase A released only traces of amino acids, and was abandoned.

C-Terminal peptides

The γ -chains and the heavy-chain-disease protein were cleaved by cyanogen bromide in 70% (v/v) formic acid, and after being freeze-dried the reaction mixture was dissolved in 5–7 ml. of 0.05 N-ammonia and fractionated by chromatography on a column

Table 2. *Amino acid compositions of the γ -chains*

	Amino acid composition (moles of amino acid/mole of γ -chain)							
	Normal human*	Daw 28†	Mar	Zuc¶	Vil	Wan	She	Ger
Ala	19	20	16	7	19	18	19	18
Arg	12	11	17	7	17	12	13	14
Asp	33	34	39	20	34	36	35	37
Cys·CH ₂ ·CO ₂ H	4	4.4	10	0	10	6	6	3
CyS	7‡	3.7	6‡	5‡	7‡	11‡	8‡	7‡
Glu	39	36	36	28	38	38	41	41
Gly	28	26	31	12	30	29	27	32
His	10	8.9	8	5	8	8	11	9
Ile	8	8.1	7	5	7	9	6	7
Leu	31	36	36	17	30	33	32	34
Lys	29	29	25	17	25	25	27	26
Met	4‡	3.6	4‡	3‡	8‡	4‡	6‡	4‡
Phe	13	13	15	9	12	14	13	16
Pro	33	34	42	23	43	32	29	31
Ser	50	47	45	22	40	48	53	50
Thr	34	35	40	15	33	37	35	29
Trp§	8	7.5	7	3	9	9	10	9
Tyr	17	17	13	5	18	15	15	20
Val	41	41	38	23	40	43	42	43
Total	420	415	435	226	428	427	428	430

* Calculated from the data of Crumpton & Wilkinson (1963).

† Press *et al.* (1966).

‡ Half-cystine and methionine were determined as cysteic acid and methionine sulphone respectively (Moore, 1963).

§ Estimated spectrophotometrically (Bencze & Schmid, 1957).

|| Based on a mol.wt. of 50 000 (Pain, 1963), and assuming a carbohydrate content of 3.6% (Müller-Eberhard, Kunkel & Franklin, 1956).

¶|| Based on a mol.wt. of 26 000 (Osserman & Takatsuki, 1964), and assuming a carbohydrate content of 7.0%.

(3.5 cm. $\times$ 90 cm.) of Sephadex G-50 in 0.05 N-ammonia at room temperature; the extinction of the eluate was measured at 215 m μ . In each case a peptide was isolated; the amino acid compositions, elution volumes (relative to the total column volume) and yields of these peptides are shown in Table 3. The peptides were confirmed as being C-terminal by the absence of homoserine and by the identification of glycine as the C-terminal amino acid by hydrazinolysis (0.5–0.7 mole/mole, uncorr.). Again, digestion of these peptides with carboxypeptidase A yielded only traces of amino acids, with the exception of those of the γ_{2d} subclass, i.e. Ger and She, which are discussed below. In several instances it was necessary to purify the C-terminal peptides further by paper electrophoresis; the mobilities of the peptides are also shown in Table 3. All the peptides were devoid of tryptophan, and in each peptide histidine was established as the N-terminal residue by Edman degradation, which gave a decrease of 0.6–0.9 mole of histidine/mole of peptide with no significant change in any other amino acid.

Sequence of Zuc and Mar C-terminal peptides

The product of the tryptic digestion of 2 μ moles of Zuc C-terminal peptide was fractionated by chromatography on a column (1.2 cm. $\times$ 145 cm.) of Sephadex G-25 in 0.02 N-ammonia; the extinction of the eluate was measured at 215 m μ . Three peaks (ZucT1–3) were obtained at 66, 73 and 85% of the total column volume respectively. When examined by paper electrophoresis at pH 6.5 and 3.5, each fraction appeared sufficiently pure to be used for the subsequent sequence studies.

ZucT1: Ser-Leu-Ser-Leu-Ser-Pro-Gly. This peptide stained yellow with ninhydrin on paper, the colour slowly changing through grey to blue with time. It possessed mobilities on high-voltage paper electrophoresis of 0 at pH 6.5 and 0.17 at pH 3.5. The peptide was recovered in 93% yield and gave the following composition in moles/mole: serine, 2.84; proline, 1.13; glycine, 1.18; leucine, 2.00. The sequence was established by five steps of the Edman degradation, as shown in Table 4. As this peptide contains the single glycine residue it must be C-terminal.

Table 3. *Amino acid compositions and characteristics of the C-terminal peptides*

	Amino acid composition (moles of amino acid/mole of peptide)							Normal human†
	Zuc	Mar	Wan	Vil	Ger	She	Daw*	
Ala	1.00	0.98	1.00	1.00	1.00	1.29	1.1	0.9
Arg	0.97	1.13	—	1.17	—	—	—	0.2
Asp	1.07	1.05	1.11	0.99	1.22	1.26	1.1	1.2
Glu	2.03	1.94	1.97	1.88	1.99	2.28	2.0	2.0
Gly	1.14	1.21	1.16	1.12	1.14	0.91	1.1	1.3
His	1.90	2.14	2.93	1.82	2.67	1.88	3.0	2.9
Ile	—	—	—	—	—	—	—	0.1
Leu	2.99	3.00	2.88	2.86	3.70	3.88	2.9	2.9
Lys	1.07	1.08	1.11	1.05	1.02	1.15	1.0	1.0
Met	—	—	—	—	—	—	—	—
Phe	1.04	0.93	—	—	—	Trace	—	—
Pro	1.08	1.06	1.11	1.01	—	—	1.0	0.9
Ser†	2.61	2.37	2.74	2.58	2.68	2.28	2.9	2.9
Thr†	0.99	1.11	1.00	0.99	1.00	1.03	1.0	1.2
Tyr	—	—	0.87	0.70	0.78	0.65	0.8	0.9
Val	0.14	—	—	—	—	—	—	0.25
Yield (mole of peptide/mole of γ -chain)	0.65	0.77	0.67	0.57	0.72	—	0.83	0.8
Column-elution volume of peptide (% of total column volume)	64	64	63	70	67	64	—	—
Mobility of peptide, relative to lysine marker, on high-voltage paper electrophoresis at pH 3.5	0.62	0.63	0.58	0.64	0.50	0.52	0.60	0.60

* Press *et al.* (1966)

† Piggot & Press (1966).

‡ Uncorrected for destruction during hydrolysis.

Table 4. *Edman degradation of the ZucT1 peptide*The amino acid presumed to be removed at each step is shown by its content being printed in **bold type**.

Step ...	Amino acid composition (moles of amino acid/mole of peptide)				
	1	2	3	4	5
Ser	1.74	1.76	1.08	0.98	0.31
Pro	1.22	0.99	1.06	0.91	0.93
Gly	1.00	1.00	1.00	1.00	1.00
Leu	2.02	1.09	1.12	0.34	0.16
Recovery (%)	90	89	87	80	110

ZucT2: His-Glu-Ala-Leu-His-Asn-Arg. This peptide stained faintly yellow with ninhydrin on paper, and also gave positive staining reactions for histidine and arginine. It possessed mobilities of 0.32 at pH 6.5 and 0.85 at pH 3.5. The peptide was recovered in 79% yield and gave the following composition: histidine, 1.79; arginine, 1.09; aspartic acid, 1.05; glutamic acid, 0.89; alanine, 1.00; leucine, 1.03. The sequence was established by five steps of the Edman degradation, as shown in Table 5.

As this peptide contains both the histidine

residues found in Zuc C-terminal peptide as well as an N-terminal histidine residue, it must occupy an N-terminal position. The mobility of ZucT2 at pH 6.5 decreased to 0.29 after step 1 of the Edman degradation and increased to 0.66 after step 2, in agreement with the removal of a basic and an acidic residue respectively. The phenylthiohydantoin of glutamic acid was identified after the second step. In view of the marked cationic mobility of the peptide after step 2 at pH 6.5, the remaining aspartic acid residue must be present as the amide.

Table 5. *Edman degradation of the ZucT2 peptide*

The amino acid presumed to be removed at each step is shown by its content being printed in **bold type**.

		Amino acid composition (moles of amino acid/mole of peptide)				
Step ...	...	1	2	3	4	5
His		1.23	1.06	0.99	0.92	0.47
Arg		1.00	1.00	1.01	0.96	1.00
Asp		1.05	1.03	1.00	1.00	0.78
Glu		1.03	0.46	0.12	—	—
Ala		1.00	0.96	0.16	0.08	—
Leu		1.08	1.07	0.79	0.37	0.15
Recovery (%)		95	97	88	104	98

Table 6. *Edman degradation of the ZucT3 and VilT3 peptides*

The amino acid presumed to be removed at each step is shown by its content being printed in **bold type**.
n.d., Not determined.

		Amino acid composition (moles of amino acid/mole of peptide)			
		ZucT2		VilT3	
Step ...	...	1	2	1	2
Lys		0.94	0.72	n.d.	0.83
Thr		0.96	0.34	0.87	0.14
Glu		1.00	1.00	1.00	1.00
Tyr		—	—	0.05	0.04
Phe		0.07	0.05	—	—
Recovery (%)		85	52	85	105

ZucT3: Phe-Thr-Gln-Lys. This peptide stained blue with ninhydrin on paper. It possessed mobilities of 0.46 at pH 6.5 and 0.60 at pH 3.5. The peptide was recovered in 85% yield, and gave the following composition: lysine, 1.06; threonine, 0.93; glutamic acid, 1.00; phenylalanine, 0.97. The sequence was established by two steps of the Edman degradation, as shown in Table 6. The glutamic acid residue must be amidated to account for the cationic mobility of the peptide at pH 6.5. The total sequence of the Zuc peptide is shown in Table 8.

The fractionation of the tryptic digest of the Mar C-terminal peptide on Sephadex G-25 again yielded three peptides (MarT1-3) whose elution volumes, amino acid compositions, mobilities on paper electrophoresis and staining characteristics were identical with those of the ZucT1-3 peptides. Similar results as those reported above for the Zuc T1-3 peptides were obtained when MarT1-3 were subjected to three steps of the Edman degradation. While it is possible that an inversion of sequence might still exist in the remaining few residues, it is highly unlikely. The sequence is presumably as shown in Table 8.

Sequence of Vil C-terminal peptide

When the tryptic digest of the Vil C-terminal peptide was fractionated on Sephadex G-25 in 0.02N-ammonia, once again three peptides were eluted at the same relative column volumes as observed for the Zuc and Mar tryptic peptides. VilT1 and VilT2 demonstrated the same electrophoretic mobility and staining characteristics as ZucT1 and ZucT2 respectively. The amino acid composition of VilT1 was: serine, 2.69; proline, 1.29; glycine, 1.08; leucine, 2.00 (yield, 74%); and that of VilT2: histidine, 1.96; arginine, 1.04; aspartic acid, 1.00; glutamic acid, 0.91; alanine, 0.96; leucine, 1.00 (yield, 94%). When subjected to five steps of the Edman degradation these peptides proved to possess sequences identical with those shown for their Zuc counterparts.

VilT3: Tyr-Thr-Gln-Lys. This peptide stained blue with ninhydrin on paper. It possessed mobilities of 0.49 at pH 6.5 and 0.62 at pH 3.5. The peptide was recovered in 95% yield, and gave the following composition: lysine, 1.07; threonine, 0.91; glutamic acid, 1.00; tyrosine, 0.72. The

sequence was established by two steps of the Edman degradation, as shown in Table 6. The VilT3 peptide differs from ZucT3 by the replacement of a phenylalanine residue by a tyrosine residue. Again the glutamic acid residue is present in the amidated form, as evidenced by the cationic mobility of the peptide at pH6.5. The total sequence of the Vil C-terminal peptide is shown in Table 8.

Sequence of Wan C-terminal peptide

Separation of the tryptic peptides of the Wan C-terminal peptide could not be effected by chromatography on Sephadex G-25 in 0.02N-ammonia, but was possible if fractionation was carried out in 1% (v/v) formic acid (Press *et al.* 1966). The WanT1 peptide was eluted at 50% of the total column volume, possessed mobilities on paper electrophoresis of 0.42 at pH6.5 and 0.79 at pH3.5 and gave an amino acid composition of: histidine, 2.79; lysine, 0.97; aspartic acid, 0.99; threonine, 0.95; glutamic acid, 1.94; alanine, 0.89; leucine, 1.00; tyrosine, 0.81. The WanT2 peptide was eluted at 62% of the total column volume, possessed mobilities of 0 at pH6.5 and 0.15 at pH3.5 and gave an amino acid composition of: serine, 2.78; proline, 0.97; glycine, 1.12; Leu, 2.00. The sequences of the WanT1 and WanT2 peptides as established by sequential Edman degradation and the properties of the products of their chymotryptic digestion were identical with those reported for the corresponding peptides of the Daw C-terminal octadecapeptide (Press *et al.* 1966) and so will not be repeated here. The complete sequence of the Wan C-terminal peptide is shown in Table 8.

Sequence of Ger and She C-terminal peptides

Once again, fractionation of the tryptic peptides was best achieved by chromatography on Sephadex G-25 in 1% (v/v) formic acid. The GerT1 peptide possessed similar elution volume, mobilities on paper electrophoresis and amino acid composition to those reported above for the WanT1 peptide. A

combination of sequential Edman degradation on the GerT1 peptide and an examination of the chymotryptic peptides established the sequence to be identical with that of the WanT1 peptide (and the corresponding Daw peptide, Press *et al.* 1966).

GerT2: Ser-Leu-Ser-Leu-Ser-Leu-Gly. This peptide was eluted at 65% of the total column volume on Sephadex G-25 in 1% (v/v) formic acid. It possessed mobilities of 0 at pH6.5 and 0.17 at pH3.5 and stained yellow with ninhydrin on paper, the colour changing through grey to blue with time. The peptide was recovered in 72% yield, and gave the following composition: serine, 2.67; glycine, 1.12; leucine, 3.00. The sequence was established by five steps of the Edman degradation, as shown in Table 7. The GerT2 peptide differs from the C-terminal heptapeptides of the other subclasses by the replacement of the penultimate proline residue by a leucine residue. Further confirmation of this replacement was obtained by carboxypeptidase A degradation of the Ger C-terminal octadecapeptide, when 0.87, 1.82 and 2.33 moles of glycine, serine and leucine respectively/mole of peptide were found after a 24hr. digestion. The lysine-serine bond shown in the complete sequence given in Table 8 appears to be resistant to hydrolysis by carboxypeptidase A.

When native or partially reduced Ger myeloma proteins were used as substrates, only traces of amino acids were released by carboxypeptidase A. The Ger γ -chain presented the further complication of insolubility at neutral pH, and digestion by carboxypeptidase A in the presence of 2M-urea again liberated no amino acids. Glycine, serine and leucine (0.52, 1.01 and 1.34 moles/50 000g. of protein respectively) were released by carboxypeptidase A in the presence of 2M-urea if the Ger γ -chain was first totally reduced and alkylated. From these enzymic studies it would appear that the C-terminus of the native molecule is not freely available to the external environment.

The C-terminal octadecapeptide of She myeloma protein, as isolated from the cyanogen bromide reaction mixture of the γ -chain on Sephadex G-50, proved to be seriously contaminated by small

Table 7. Edman degradation of the GerT2 peptide

The amino acid presumed to be removed at each step is shown by its content being printed in **bold type**.

		Amino acid composition (moles of amino acid/mole of peptide)				
Step	...	1	2	3	4	5
Ser	...	1.98	1.79	1.13	0.73	0.35
Gly		1.20	1.13	1.00	1.00	1.00
Leu		3.00	2.00	2.05	1.25	0.87
Recovery (%)		89	72	65	75	57

peptides containing no homoserine. As none of the other myeloma proteins exhibited extensive lability under the conditions of cyanogen bromide cleavage employed here, it was thought to be more likely that this represented degradation accompanying storage (Robert, Denes & Crepin, 1965), this serum having been collected some 8 years previously. In agreement with this interpretation was the liberation of a spectrum of neutral amino acids by the action of carboxypeptidase A on the She γ -chain in the presence of 2M-urea. The amino acid composition of the She C-terminal octadecapeptide reported in Table 3 was obtained on material that had been purified by electrophoresis on paper, a step that is unfortunately accompanied by a 50–65% loss of peptide and which precluded extensive sequence studies. The tryptic peptides SheT1 and SheT2, also purified by paper electrophoresis, had mobilities, staining characteristics and amino acid compositions similar to their Ger counterparts. In particular, SheT2 contained an additional residue of leucine (i.e. 3 moles/mole of glycine) and no proline, which is consistent with the observed leucine-for-proline substitution in the GerT2 peptide. Although its sequence is not firmly based on equivalent Edman-degradative data, it is highly probable that the She C-terminal octadecapeptide possesses a sequence identical with that established for the Ger C-terminal octadecapeptide (Table 8).

DISCUSSION

The marked homology within the C-terminal octadecapeptides isolated from the myeloma γ -chains of various subclasses and Gm specificities shown in Table 8 is immediately obvious. Despite

the relatively small number of proteins investigated, it appears probable that the primary structure of the C-terminus of the γ_{2c} subclass is to be characterized by the arginine-for-histidine substitution in position C12, and that of the γ_{2d} subclass by the leucine-for-proline substitution in position C2. The latter replacement is not likely to be detected on 'fingerprints' of the tryptic digests of Fc fragment, but the γ_{2c} subclass substitution (arginine for histidine) introduces a new point of tryptic cleavage and the formation of two new peptides that should distinguish it from the γ_{2a} , γ_{2b} and γ_{2d} subclasses. Indeed, this was probably the cause of some of the differences observed in the 'fingerprint' studies of Fc fragments prepared from γ_{2c} myeloma proteins when compared with those prepared from the other subclasses (Frangione *et al.* 1966). The phenylalanine-for-tyrosine substitution in position C11 in the γ_{2c} subclass appears at this time to be related to the change of Gm specificity, i.e. Gm(5+, 13+, 14+, 21-) versus Gm(5-, 13-, 14-, 21+). The replacement does not appear to be directly associated with antigenic specificity, however, as the Vil and Zuc C-terminal octadecapeptides were unable to inhibit agglutination when incorporated into the system employed to determine the Gm types of proteins (H. G. Kunkel, unpublished work). Further investigations of myeloma proteins of the γ_{2c} subclass of rarer phenotypes should help to clarify the significance of this observation. These replacements in the C-terminal octadecapeptide of the γ_{2c} subclass were first recognized in the Zuc urinary heavy-chain-disease protein (Prahl, 1966). The observation provides conclusive chemical evidence that the biosynthesis of this myeloma variant (Osserman & Takatsuki, 1964) proceeded

Table 8. C-Terminal sequences of human and several non-human γ -chains

Amino acid replacements between different human γ -chains are shown in **bold type**; ones between γ -chains of different species, in *italics*.

Residue no. ...	C19	C15	C10	C5	C1
Daw*	}	Met-His-Glu-Ala-Leu-His-Asn- His -Tyr-Thr-Gln-Lys-Ser-Leu-Ser-Leu-Ser- Pro -Gly			
Normal human†					
Wan	}	Met-His-Glu-Ala-Leu-His-Asn- Arg -Phe-Thr-Gln-Lys-Ser-Leu-Ser-Leu-Ser- Pro -Gly			
Zuc					
Mar	}	Met-His-Glu-Ala-Leu-His-Asn- Arg -Tyr-Thr-Gln-Lys-Ser-Leu-Ser-Leu-Ser- Pro -Gly			
Vil					
She	}	Met-His-Glu-Ala-Leu-His-Asn- His -Tyr-Thr-Gln-Lys-Ser-Leu-Ser-Leu-Ser- Leu -Gly			
Ger					
Rabbit‡		Met-His-Glu-Ala- <i>Leu</i> -His-Asn-His-Tyr-Thr-Gln-Lys-Ser- <i>Ile</i> -Ser- <i>Arg</i> -Ser-Pro-Gly			
Horse IgG§		Met-His-Glu-Ala- <i>Leu</i> -His-Asn-His-Tyr-Thr-Gln-Lys-Ser- <i>Val</i> -Ser- <i>Lys</i> -Ser-Pro-Gly			
Horse Ig(T)§		Met-His-Glu-Ala- <i>Val</i> -Glu-Asn-His-Tyr-Thr-Gln-Lys- <i>Asn</i> - <i>Val</i> -Ser- <i>His</i> -Ser-Pro-Gly			

* Press *et al.* (1966).

† Piggot & Press (1966).

‡ Givol & Porter (1965).

§ Weir, Porter & Givol (1966).

to a termination that has a corollary in intact heavy chain, and implies that the cistrons transcribed in the biosynthesis of the C-terminal region of myeloma proteins and this variant are structurally identical.

The C-terminal sequences of myeloma proteins of the γ_{2a} and γ_{2b} subclasses were identical; however, one must bear in mind that only one γ_{2a} myeloma protein was examined here, and the Gm(n) phenotype of that was unknown (Kunkel *et al.* 1966). Further, only one myeloma protein of subclass and Gm type γ_{2b} :Gm(1+) has been examined (Press *et al.* 1966). But on the basis of the high recovery of the Daw-like peptide recovered from normal human IgG (0.8 mole/mole; Piggot & Press, 1966), and the probability that the IgG was Gm(1+, 4+), it is likely that this sequence is characteristic of the proteins of the γ_{2b} subclass. This conclusion is supported by the observations that the antigenic determinant of Gm(4) appears to be in the Fd area of the γ -chain (Gold, Mandy & Fudenberg, 1965), and by the absence of obvious differences other than the Gm(1)-non-Gm(1) peptides in the 'fingerprints' of the Fc fragments of the many γ_{2b} myeloma proteins investigated (Frangione *et al.* 1966).

It is noteworthy that Piggot & Press (1966) did not observe the homologous sequences of the various subclasses in their investigation of the C-terminal peptide of the γ -chain of normal human IgG. If one considers the relative proportion of the various subclasses in normal human IgG the reason becomes obvious (γ_{2a} , 8–10%; γ_{2b} , 70–80%; γ_{2c} , 6–8%; γ_{2d} , 2–4%). This emphasizes the caution that must be exercised in the interpretation of studies on the primary structure of the immunoglobulins. Indeed, in view of the variation in methionine content of the γ -chains of the myeloma proteins (Table 2), it is fortunate that the methionine in position C19 appears invariant. This obviously cannot be the case with all the methionine positions, as indicated by the studies of the Gm(1) versus non-Gm(1) region by Thorpe & Deutsch (1966). The uniqueness of the methionine in position C19 has also been demonstrated in the rabbit (Givol & Porter, 1965) and horse (Weir *et al.* 1966) γ -chains, the C-terminal octadecapeptide sequences of which are also shown in Table 8 for purposes of comparison. The interspecies homologies are as striking as are the intraspecies. All the amino acid replacements can be accounted for by single-nucleotide substitutions in the triplet code (Morgan, Wells & Khorana, 1966).

The existence of four genetic loci or cistrons determining the γ -chains of IgG has been suggested on the basis of the identification of the four subclasses of γ -chain, and recognition of the linkage of the Gm specificities with certain of these subclasses (Kunkel *et al.* 1964; Mårtensson, 1966). The

data presented in the present paper are in accord with this view, necessitating the existence of at least three unique loci to account for the maintenance of the three unique structural variations, i.e. γ_{2a} – γ_{2b} , γ_{2c} and γ_{2d} . Though this limited stretch of sequence does not necessitate the independent existence of loci for the γ_{2a} and γ_{2b} subclasses, the demonstrated linkage of Gm(n) to the γ_{2a} subclass (Kunkel *et al.* 1966) and the distinct difference of biological properties of the γ_{2a} and γ_{2b} myeloma proteins (Terry, 1966) implies that this is probably the case. In addition, the homology of the C-terminal sequences of the subclasses is most adequately explained by gene duplication of a primordial cistron. It is tempting to postulate the cistron of the present γ_{2a} – γ_{2b} subclass as the point of subclass emergence based on the predominance of this subclass in the IgG and the close resemblance of the C-terminal octadecapeptide of this subclass to its rabbit and equine counterparts. Considerably more knowledge of primary structure and comparative sequences must be gained, however, before the order of emergence of the subclasses will become clear.

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