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Amino Acid Sequence of the V_H Region of Human Myeloma Cryoimmunoglobulin IgG Hil†

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ABSTRACT: We have determined the complete amino acid sequence of the variable region of the heavy chain (V_H) from a human myeloma cryoimmunoglobulin, IgGl Hil. The N-terminal sequence was determined by automatic procedures after specific removal of the α-amino terminal pyroglutamic acid by calf liver pyroglutamate aminopeptidase. This deblocking procedure was applied to whole heavy chain and to a heavy chain fragment (positions 1–99) derived by acid treatment of Fab Hil. CNBr fragments spanning residues 35–81 and 84–256 provided two additional starting points for automatic sequencing. The results of these automatic runs gave the sequence from residues 35 to 70 and from 84 to 117. Characterization and sequencing of tryptic peptides were used to complete the C-terminal end sequence of one CNBr fragment (positions 35–81) and to find the sequence of a gap between the two CNBr fragments. The sequence toward the C-terminal end of variable region and the beginning of the constant region was determined on a tryptic peptide spanning residues 99–125. This peptide was sequenced to its C terminus by automatic liquid-phase procedures in the presence of polybrene. In addition, tryptic peptides covering the whole extent of the variable region were isolated and characterized to provide further support to the automatic sequence data. The proposed sequence of the variable region indicates that it may be assigned to subgroup V_HIII. The V_L sequence of IgG Hil has been reported [J. A. Lõpez de Castro et al. (1978) *Biochemistry 17*, 1718–1723]. A study of the three-dimensional structure of the Fab fragment from this protein by X-ray diffraction is in progress.

IgG[1] Hil is a human myeloma cryoimmunoglobulin, the Fab fragment of which has been crystallized (Rossi & Nisonoff, 1968; Humphrey et al., 1969). X-ray diffraction studies of this fragment are in progress in our laboratory. A necessary step in these studies is the determination of the amino acid sequence, which will provide information required for a complete interpretation of a high-resolution Fourier map of Fab Hil.

The three-dimensional structure and the amino acid sequence of the Fab fragment of another human immunoglobulin, IgG New, have been reported (Poljak et al., 1973, 1974, 1977; Chen & Poljak, 1974). Studies on IgG Hil were undertaken with the aim of correlating the amino acid sequence at the hypervariable regions with the conformation of its combining site. It is expected that a comparison between IgG New and IgG Hil will contribute to our understanding of the relation between structure of the antigen combining site and antibody specificity.

We have already reported the amino acid sequence of the variable region of the L(λ) chain of IgG Hil (Lõpez de Castro et al., 1978). In this paper we present the complete amino acid sequence of the variable region (V_H) of its heavy chain, Figure 1.

Materials and Methods

Preparation of H Chain Hil. Human myeloma immunoglobulin Hil (IgGl, a cryoglobulin, Gm (1+, 3–, 4–, 5–), λL chain) was purified from serum as previously described (Rossi & Nisonoff, 1968). Preparative separation of H and L chains was carried out after mild reduction and carboxymethylation of purified IgG as described before for IgG New (Chen & Poljak, 1974).

Preparation of CNBr Fragments. Lyophilized, mildly reduced, and alkylated H chains were cleaved by CNBr as described (Gross, 1967). The CNBr-treated protein was fractionated by gel filtration on a Sephadex G-100 column (5 × 90 cm) equilibrated with 1 M acetic acid. Three peaks were observed by this procedure (Figure 2A). Peak I contained the N-terminal peptide H1 (positions 1–34) cross-linked to the fragment H3 (positions 84–256) by a disulfide bridge.

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[1] Abbreviations used: for immunoglobulins, their polypeptide chains and fragments are as recommended [(1964) *Bull. W.H.O. 30*, 447]; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; LC, high-performance liquid chromatography; Pth, phenylthiahydantoin; DMAA, dimethylallylamine; PITC, phenyl isothiocyanate; PCA, pyrrolidonecarboxylic acid.

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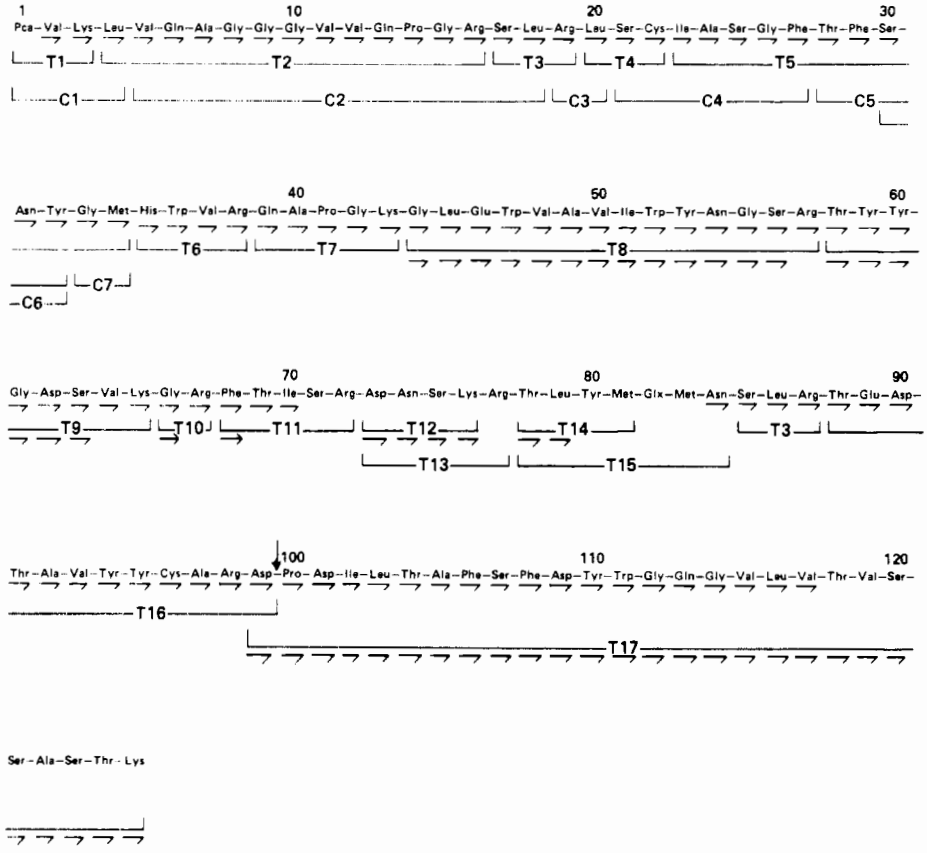

FIGURE 1: Amino acid sequence of the V_H region of IgG Hil. Half arrows indicate residues which were determined by automatic Edman degradation of (a) deblocked N-terminal fragment obtained after mild acid hydrolysis (positions 2–34); (b) CNBr fragment H2 (positions 35–70); (c) CNBr fragment H3 (positions 84–117); (d) tryptic peptide T 17 (positions 99–125). Half arrows under peptides T8, T9, T12, and T14 indicate the residues in these peptides which were determined by automatic sequencing. Full arrows represent residues in T10 and T11 which were identified by dansyl N-terminal analysis. Tryptic (T) and chymotryptic peptides (C) that were characterized are indicated. The vertical arrow between Asp(99) and Pro(100) indicates the site of acid cleavage.

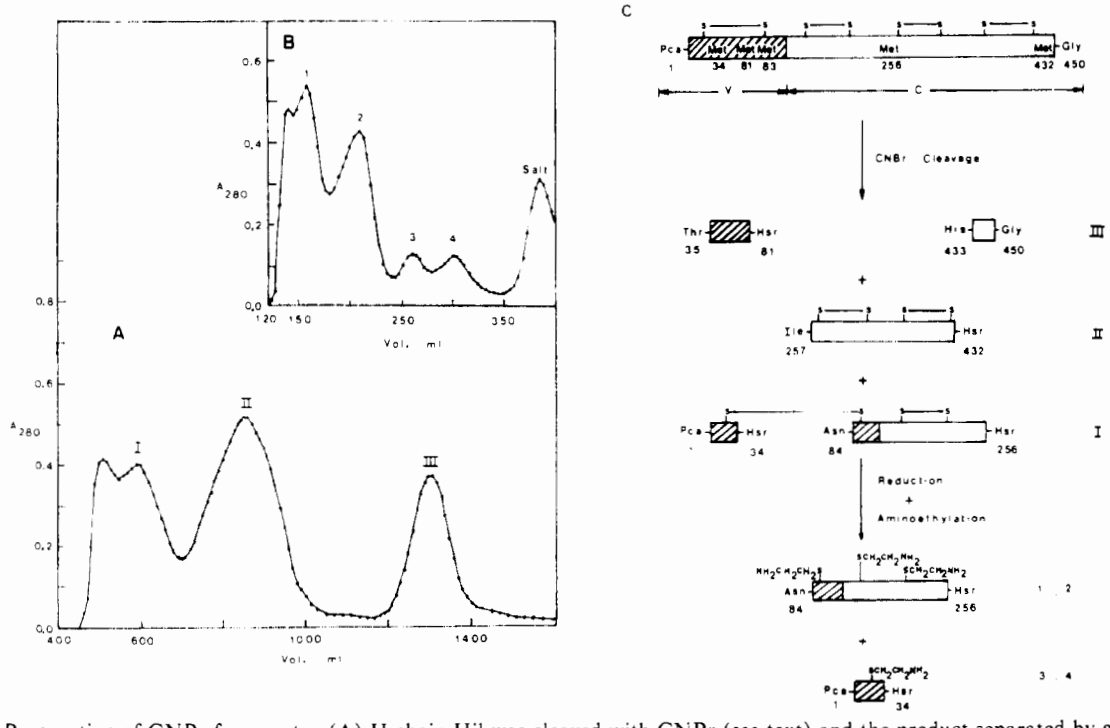

FIGURE 2: Preparation of CNBr fragments. (A) H chain Hil was cleaved with CNBr (see text) and the product separated by gel filtration in a 5.0 × 90 cm column of Sephadex G-100 equilibrated with 1 M acetic acid and fitted with upflow adaptors. (B) Peak I of A was totally reduced and aminoethylated (see text). The reduced material was fractionated on a 2.5 × 90 cm column of Sephadex G-100 equilibrated with 1 M acetic acid. (C) Scheme for the CNBr cleavage of H chain Hil. The location of the disulfide bridges is shown as well as the correlation of the fragments with the elution patterns shown in A and B.

Table I: Amino Acid Composition of the Fragments from the Heavy Chain and Fab' of IgG Hil[a]

	CNBr fragments from H chain			acid-cleaved fragment	tryptic fragment
	III[b]	2[b]	4[b]	III[e]	T17[f]
Asp	4.5 (4)[c]	13.0 (13)	1.4 (1)	8.5 (8)	3.0 (3)
Thr	3.7 (3)	16.2 (18)	1.2 (1)	6.7 (6)	3.0 (3)
Ser	4.9 (4)	18.7 (23)	2.9 (4)	8.0 (9)	4.3 (4)
Glu	3.6 (2)	8.5 (7)	3.2 (3)	8.4 (7)	1.3 (1)
Pro	2.0 (1)	16.2 (18)	1.5 (1)	2.8 (2)	2.0 (1)
Gly	6.3 (5)	11.7 (12)	5.4 (6)	10.3 (11)	2.7 (2)
Ala	2.0 (2)	9.8 (10)	2.1 (2)	6.5 (6)	2.0 (2)
Cys[d]		2.3 (6)	(1)	(2)	0.1 (0)
Val	4.8 (4)	18.7 (17)	3.8 (4)	8.8 (9)	2.9 (3)
Met	(1)	(1)	(1)	2.1 (3)	
Ile	1.8 (2)	2.8 (2)	1.0 (1)	3.1 (3)	0.8 (1)
Leu	3.9 (2)	14.2 (15)	2.8 (3)	5.4 (6)	2.9 (2)
Tyr	4.1 (4)	6.9 (6)	1.3 (1)	6.5 (7)	1.8 (1)
Phe	1.1 (1)	6.6 (7)	1.8 (2)	2.5 (3)	1.6 (2)
His	1.4 (1)	3.2 (3)		1.0 (1)	0.5 (0)
Lys	3.5 (3)	11.8 (10)	1.0 (1)	4.6 (4)	0.9 (1)
Trp	(3)	(2)		(3)	(1)
Arg	5.0 (5)	3.5 (3)	2.1 (2)	8.3 (9)	
residues					
major	35–81	84–256	1–34	1–99	99–125
minor	433–450				152–209

[a] Values listed as residues per mol of the major fragment. [b] Symbols as used in Figure 2. [c] Numbers in parentheses are the assumed integral values of the major fragment. [d] Detected as aminoethylcysteine, values not corrected for destruction. [e] Symbol as used in Figure 3. [f] See text (Materials and Methods).

Peak II contained fragment H4 spanning residues 257 to 432. Peak III contained two fragments, H2 (positions 35–81) and H5 (positions 433–450). The dipeptide (residues 82–83) Glx-homoserine lactone was not detected in this chromatographic separation. Peak I was totally reduced by dissolving the protein in 7 M guanidine–0.24 M β-mercaptoethanol and 0.02 M Tris, pH 8.0, and incubating for 4 h at room temperature. The reduced material was aminoethylated as described (Cole, 1967). After this treatment, samples were loaded on a 2.5 × 90 cm column packed with Sephadex G-100 in 1 M acetic acid. Four peaks were observed (Figure 2B). Peak 1 contained aggregated material from fragment H3, and peak 2, the monomer of H3. Peak 3 contained aggregated material from fragment H1, and peak 4, the monomer of H1. The overall scheme is illustrated in Figure 2C. Their amino acid compositions are presented in Table I.

Preparation of Fragments by Mild Acid Hydrolysis. Fab fragment was obtained from IgG Hil by papain digestion in the absence of reducing agents (Stanworth & Turner, 1973) and purified as described (Rossi & Nisonoff, 1968). The final preparations did not show any significant contamination with undigested IgG Hil as judged by gel filtration analysis. Fab preparations were dissolved (12 mg/mL) in 70% (v/v) formic acid; N_2 was bubbled through the solution in order to avoid oxidative cleavage of disulfide bonds. The samples were then incubated at 37 °C for 32 h. After this period, the reaction mixture was diluted in 10 volumes of distilled water and freeze-dried. This material was fractionated by gel filtration through a column (2.5 × 90 cm) filled with Sephadex G-100 and equilibrated with 1 M acetic acid. The procedure allowed the isolation of a fragment (positions 1–99) containing the amino-terminal portion of the H chain and including most of the V_H region (peak III, see Figure 3). This fragment was completely reduced by treatment with 6 M guanidinium hydrochloride, 0.1 M dithiothreitol, 2 mM EDTA, and 0.2 M Tris-HCl buffer, pH 8.1, for 4 h at 45 °C. Carboxymethylation of the reduced fragment was achieved by adding iodoacetic acid at a final concentration of 0.3 M and incubating for 30 min in the dark. The amino acid composition of this fragment is presented in Table I.

Deblocking of the Amino Terminus. Removal of the amino-terminal pyrrolidonecarboxylic acid (PCA) was carried out using calf liver pyroglutamate aminopeptidase (L-pyroglutamyl-peptide hydrolase, EC 3.4.11.8, Boehringer Mannheim Biochemicals) under previously described conditions (Podell & Abraham, 1978). PCA could be successfully cleaved from the complete H chain or from an N-terminal fragment (positions 1–99) obtained by partial acid hydrolysis.

Preparation of Tryptic and Chymotryptic Peptides. Tryptic and chymotryptic digestions as well as high voltage paper electrophoresis and paper chromatography were carried out as described before (Poljak et al., 1977). Preparative amounts of some tryptic peptides were obtained as follows. Thirty milligrams of CNBr fragment H2 (residues 35–81) was digested overnight with trypsin. The insoluble product, T8 (Figure 1), was separated by centrifugation, washed three times with 0.1 M NH_4HCO_3 buffer, pH 9.1, and analyzed separately. The soluble fraction, containing 16 mg of material, was fractionated by ion-exchange chromatography on a column (0.6 × 70 cm) filled with Beckman A-15 resin. The procedure and conditions were as described for a Dowex Ag50-X2 column by Chen et al. (1978). The effluent of the column was monitored by the ninhydrin reaction after alkaline hydrolysis of 0.2 mL of each of the collected 3-mL fractions (Moore & Stein, 1954).

In another experiment 19 mg of the N-terminal piece obtained by partial acid hydrolysis of Fab fragment (peak III in Figure 3) was digested with trypsin as above. The soluble fraction of the digested material was fractionated in the same ion-exchange column, using a different pH gradient (Brown et al., 1974) and a gradient making chamber with five compartments of 250 mL each. The analysis of the effluent was carried out as above.

CNBr fragment H3 (residues 84–256) was digested overnight with trypsin. The insoluble product containing peptide T17 (Figure 1) and a constant region peptide spanning residues 152–209 (Table I) was washed several times with 0.1 M NH_4HCO_3 and dissolved in 1 M acetic acid. This sample was fractionated by gel filtration through Sephadex G-50 equilibrated with 1 M acetic acid. The peak immediately following the breakthrough contained 83% T17 and 17% constant region peptide as judged by quantitation of results after automatic sequencing of this material.

Sequence Determination. Automated Edman degradations (Edman & Begg, 1967) were carried out in a 890C Beckman sequencer. The Beckman protein 1 M QUADROL program no. 122974 and the Beckman peptide DMAA program no. 102974 were generally used for sequencing big polypeptide fragments and peptides, respectively. Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide; Aldrich Chemical Co., Milwaukee, WI) was used in some cases. About 200 nmol of a given peptide sample was mixed with 0.1 mL of an aqueous solution containing 30 mg/mL of polybrene (Klapper et al., 1978). The mixture was loaded into the spinning cup of the sequencer and the automatic Edman degradation was carried out with the DMAA program without PITC delivery during the first two cycles.

Identification of Pth-amino Acids. The products of sequential Edman degradations were identified by high pressure liquid chromatography (LC, Zimmerman et al., 1977), HI hydrolysis or NaOH hydrolysis followed by amino acid analysis

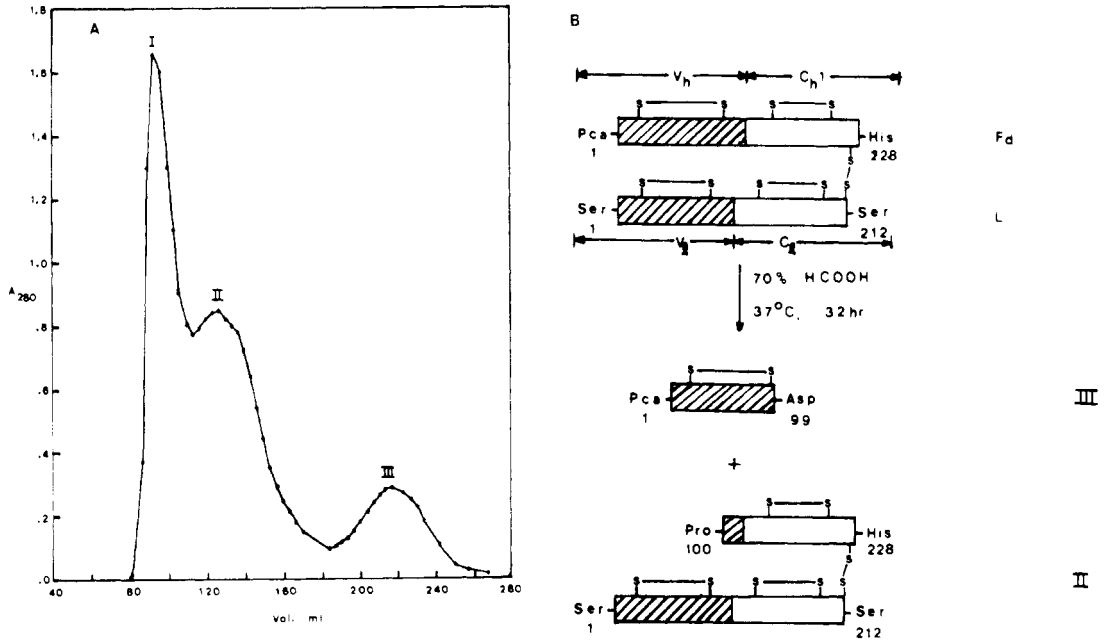

FIGURE 3: Separation of fragments obtained after mild acid hydrolysis of the Fab fragment from IgG Hil. (A) The Fab fragment was incubated with 70% (v/v) formic acid for 32 h at 37 °C (see text). The product was separated by gel filtration in a 2.5 × 90 cm column filled with Sephadex G-100 and equilibrated with 1 M acetic acid. (B) Scheme for the acid cleavage of the Fab fragment. The location of the disulfide bridges is shown as well as the correlation of the fragments with the elution pattern shown in A.

(Smithies et al., 1971) and, in some cases, by thin-layer chromatography (Summers et al., 1973). The general methodology was as described in a previous report (López de Castro et al., 1978). Quantitation of automatic runs was obtained either by LC or by amino acid analysis after HI hydrolysis of the Pth-amino acids.

Amino Acid Analyses. These were carried out using a Durrum D-500 amino acid analyzer, equipped with a ninhydrin detection system.

Results

The general approach used to determine the sequence of V_H Hil was the isolation of large polypeptide fragments obtained by chemical cleavage of the H chain, followed by automatic sequencing of the purified products. The positions which were not established by this approach were determined after isolation, characterization, and automated sequencing of the corresponding tryptic peptides and by peptide overlapping.

Two procedures were used to obtain fragments of the H chain suitable for automatic Edman degradation: CNBr cleavage and mild acid hydrolysis (see Materials and Methods). Three CNBr fragments, H1 (residues 1–34), H2 (residues 35–81), and H3 (residues 84–256), covered the whole variable region with the exception of the dipeptide Glx-Met (residues 82–83) which was not detected by the fractionation procedure. Fragments H2 and H3 were submitted to automatic Edman degradation. Attempts to submit either the N-terminal CNBr fragment H1 or the complete heavy chain to automated Edman degradation were consistently unsuccessful, suggesting that the N terminus of the H chain was chemically blocked. The automatic sequencing of CNBr fragment H3 revealed the existence of a sequence Asp-Pro at positions 99–100. Aspartylproline peptide bonds are known to be particularly susceptible to acid hydrolysis under relatively mild conditions (Piszkiewicz et al., 1970). Since both the L chain and the C_H1 domain of IgG Hil do not have any such bond (López de Castro et al., 1978), a procedure was designed to obtain an N-terminal fragment of the heavy chain (positions 1–99) by mild acid hydrolysis using Fab Hil as starting material (see Materials and Methods and Figure 3). This H-chain N-terminal fragment was used for automatic sequence determination after deblocking of the amino terminus with calf liver pyroglutamate aminopeptidase. This enzyme specifically hydrolyzes peptide bonds involving pyrrolidonecarboxylic acid (PCA) residues. From the quantitation of Pth-amino acids after automatic Edman degradation, the yield of the deblocking reaction was estimated to be about 80%. Intact H chain was also successfully deblocked with the same enzyme and thus made suitable for automatic Edman degradation. However, the background was significantly higher than that obtained in the sequencing of the deblocked N-terminal piece (residues 1–99), thus reducing the number of cycles to which the run could be extended. The increased background, which presumably arises from random hydrolysis of the polypeptide chain during the Edman reaction, can be accounted for by the much greater length of the intact H chain as compared with that of the N-terminal piece.

The automatic sequence of the deblocked N-terminal piece was unambiguously extended to Met-34 (Figure 4A). Tryptic peptide T5 and chymotryptic peptides C5, C6 and C7 (see Tables II and III) were obtained after digestion of CNBr fragment H1, and their amino acid composition was determined in order to provide further support for the residues determined toward the end of the automatic sequencer runs.

CNBr fragment H2 was most conveniently sequenced by using the QUADROL program during the first 18 cycles and then switching to the DMAA peptide program. This procedure was found to minimize the interference produced by the C-terminal octadecapeptide (H5, residues 433–450) which coeluted with H2 during the isolation of this fragment. A drop in the absolute yield of the Pth-amino acids was evident immediately after switching from the QUADROL to the DMAA program, but no significant alteration of the repetitive yield was observed. With this procedure, the run was extended to 36 cycles (positions 35–70, Figure 4B). In addition, tryptic peptides covering the whole length of the fragment (positions 35–81) were obtained and characterized (peptides T6 to T14; see Table I). T8 was an insoluble peptide which precipitated

Table II: Amino Acid Composition of the Tryptic Peptides of V_H Hil[a]

AA[b,k]	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17[j]
Asp					1.4 (1)		0.5[f] (0)	0.9 (1)	1.0 (1)			1.8 (2)	1.9 (2)		0.9 (1)	2.4 (2)	2.5 (3)
Thr					0.8 (1)			0.4 (0)	0.6[e] (1)		0.9 (1)			0.6[e] (1)	0.7 (1)	1.8 (2)	2.3 (3)
Ser			0.9 (1)	1.0 (1)	1.2 (2)		0.2 (0)	1.1 (1)	0.6 (1)		0.7 (1)	1.0 (1)	0.7 (1)		1.0 (1)	1.2 (1)	2.9 (4)
Glu	1.0 (1)	2.3 (2)					1.0 (1)	1.1 (1)	0.3 (0)								0.8 (1)
Pro		1.0 (1)		0.3 (0)			0.8 (1)	0.3 (0)									1.2 (1)
Gly		3.6 (4)			2.3 (2)		1.0 (1)	2.2 (2)	1.2 (1)	1.1 (1)					0.4 (0)	0.5 (0)	2.0 (2)
Ala		1.1 (1)			0.9 (1)		0.8 (1)	1.1 (1)								2.0 (2)	1.8 (2)
Cys				0.2[d] (1)												0.6[e] (1)	
Val	1.0 (1)	2.4 (3)				1.0 (1)		1.6 (2)	1.0 (1)							1.1 (1)	2.0 (3)
Met					nd[i] (1)									(1)[h]	2.0 (2)		
Ile					0.4[c] (1)			0.6 (1)			1.0 (1)						0.7 (1)
Leu		0.4[c] (1)	1.0 (1)	0.3[c] (1)				1.0 (1)						1.0 (1)	1.0 (1)		2.0 (2)
Tyr					1.0 (1)			1.2 (1)	1.8 (2)					1.1 (1)	1.1 (1)	1.6 (2)	1.2 (1)
Phe					1.9 (2)			0.3 (0)			0.9 (1)						1.4 (2)
His						0.2[c] (1)											
Lys	0.3[c] (1)						1.1 (1)		0.5[c] (1)			1.2 (1)	0.6[c] (1)				0.9 (1)
Trp						nd[i] (1)		(2)[g]									nd[i] (1)
Arg		0.8 (1)	0.9 (1)			1.0 (1)		1.1 (1)		0.9 (1)	1.1 (1)		1.0 (1)			0.8 (1)	
total	3	13	3	3	12	4	5	14	8	2	5	4	5	4	7	12	27
residue no.	1–3	4–16	17–19	20–22	23–34	35–38	39–43	44–57	58–65	66–67	68–72	73–76	73–77	78–81	78–84	88–99	99–125

[a] Values listed as residues per mole. Numbers in parentheses are the assumed integral values. [b] Experimental values not corrected for destruction or for incomplete hydrolysis. [c] Partially destroyed by ninhydrin. [d] Detected as aminoethylcysteine. Value not corrected for destruction. [e] Detected as cysteic acid. Value not corrected. [f] Peptide T7 is contaminated with about 25% of peptide T12, for which the chromatographic and electrophoretic behavior are very similar. [g] Qualitatively detected. [h] Qualitatively detected as homoserine. [i] Not determined. [j] Values corrected for contamination with 17% of the constant region peptide spanning residues 152–209. [k] AA = amino acid.

selectively during tryptic digestion of H2. T9 was obtained by ion-exchange chhomatography. Both peptides were automatically sequenced (Figure 1). T12 and T14 were isolated in good yield by ion-exchange chromatography and were automatically sequenced as well, thus providing direct sequence information at the carboxy-terminal portion of CNBr fragment H2. The straightforward alignment of the tryptic peptides T8 to T14 established the complete sequence of the CNBr fragment H2 (Figure 1).

Isolation and characterization of tryptic peptides covering most of the variable region was carried out after tryptic digestion of the N-terminal fragment (residues 1–99) obtained by mild acid hydrolysis. This approach allowed us to isolate a peptide, T15, which contained two methionine residues. The sequence Glx-Met between the C-terminus of CNBr fragment H2 and the N terminus of fragment H3 was firmly established by two criteria. The first was the result of comparing the amino acid composition of peptide T15 with T14. The second was that Asn, not Glx, was found as the N terminus of the CNBr fragment H3. Thus the sequence at positions 81 to 84 should be Met-Glx-Met-Asn and not the alternative Met-Met-Glx-Asn. The Glx residue (position 82) appeared to be in the acidic form in peptide T15 as judged by its electrophoretic mobility, a fact that cannot be taken as definite for assignment of the residue, since the preparation of this peptide involved strongly acidic conditions under which extensive deamination is to be expected. The unusual carboxy terminus of T15 probably reflects a secondary point of acidic cleavage at the bond Asn^{84}-Ser^{85} during the preparation of the parental fragment (Schultz, 1967).

Automatic Edman degradation of CNBr fragment H3 was carried out to determine the amino acid sequence at the C-terminal portion of V_H (Figures 1 and 4). In this way, positions 84–117 were established. The sequence determination was extended to the end of V_H and into the C_H1 region after isolation and sequencing of tryptic peptide T17 contaminated with 17% of a constant region fragment (positions 152–209) (see Materials and Methods). Automatic Edman degradation of peptide T17 (0.9 mg of material) in the presence of polybrene (3 mg, see Materials and Methods) allowed sequencing this peptide to its C terminus, corresponding to position 125 (Figures 1 and 4). Contamination of T17 by the constant region fragment did not cause any difficulty in assigning amino acid residues at each cycle.

Discussion

The amino acid sequence of the heavy chain variable region of IgG Hil was determined in parallel with X-ray diffraction studies of its Fab fragment. The information presented in this report, as well as the amino acid sequence of the L (λ) chain (López de Castro et al., 1978), is required for the complete interpretation of a high-resolution Fourier map of Fab Hil, currently in preparation in this laboratory. In addition, the results obtained with V_H Hil add to the existing knowledge about the extent and pattern of sequence diversity exhibited by human V_H regions.

Automation has assumed a central role in protein sequencing procedures. This is mainly due to the availability of suitable programs for automatic Edman degradation of both large polypeptides and smaller peptides as well as to the development of LC systems which provide high sensitivity and resolution for the quantitative analysis of Pth-amino acids. The amino acid sequence of V_H Hil has been determined following the general strategy of isolating a few large fragments obtained by selective cleavage of the immunoglobulin molecule which could be sequenced automatically. In addition, we have

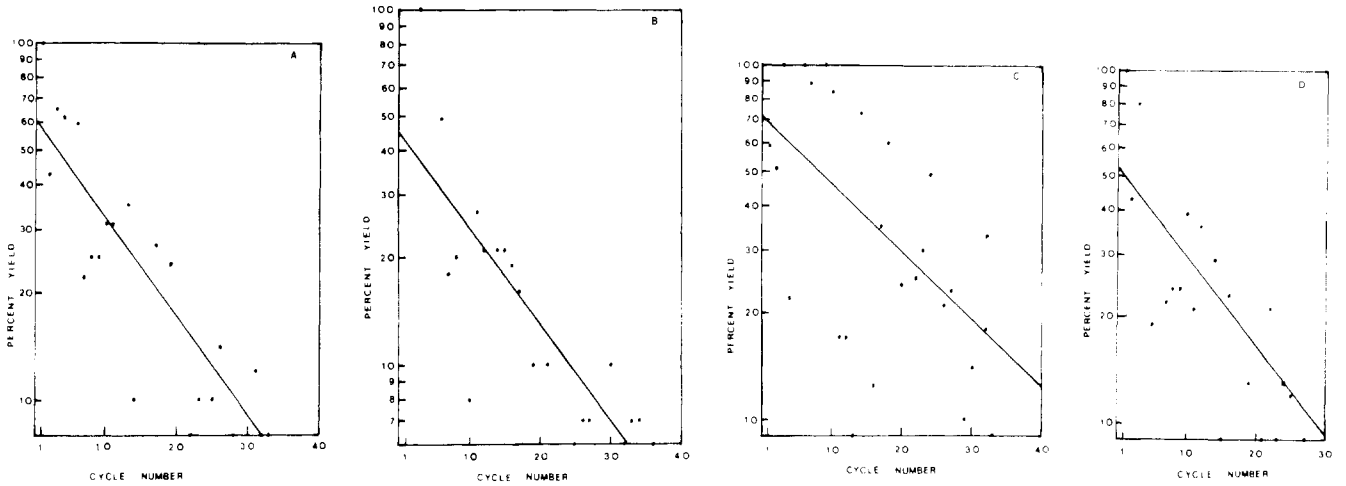

FIGURE 4: (A) Semilog plot of percent yields of Pth-amino acids from automatic Edman degradation of a deblocked N-terminal fragment spanning residues 1–99 of H chain Hil. Four milligrams of material was sequentially degraded in a Beckman 890C sequencer using the protein QUADROL program no. 122974. Quantitative yields of Pth-amino acids were obtained from LC data. The yield of the first cycle product was normalized to 100%. The line shown in this figure was fitted to the points by a least-squares regression. The repetitive yield was calculated to be 93%. (B) Semilog plot of percent yields of Pth-amino acids from automatic degradation of CNBr fragment H2 (2.5 mg). Experimental conditions were as in A except that the protein QUADROL program was used during the first 18 cycles and then the sequencing was continued with DMAA program no. 102974. The repetitive yield after cycle 19 is normalized to the relative yield in this cycle to account for losses of material due to the change of program. The average repetitive yield was 91%. (C) Semilog plot of percent yields of Pth-amino acids from automatic degradation of CNBr fragment H3 (4 mg). Experimental conditions were as in A, except that the DMAA program was used. Quantitation of results was obtained from amino acid analysis data after hydrolysis of the Pth-amino acids with HI. Since conversion values for Pth-Asn and Pth-Ser (first and second cycle) were low, yields were normalized to 100% at the third cycle. The average repetitive yield was 92%. (D) Semilog plot of percent yields of Pth-amino acids from automatic degradation of tryptic peptide T17 (0.9 mg). Conditions were as in A. The DMAA program and polybrene (see text) were used. Results were quantitated from amino acid analyses. The average repetitive yield was 94%. Percent yields for the contaminating fragment (positions 152–209) were 20 and 13 for the first and third cycles, respectively, 9 for the second, fourth, and sixth cycles, and less than 9 for the rest.

Table III: Amino Acid Composition of the Chymotryptic Peptides of CNBr Fragment H1[a]

amino acid[b]	C1	C2	C3	C4	C5	C6	C7
Asp					1.1 (1)	1.0 (1)	
Thr					0.4[c] (1)		
Ser	0.3 (0)	0.9 (1)		1.6 (2)	1.0 (1)	0.7 (1)	
Glu	1.1 (1)	2.2 (2)			0.4 (0)	0.2 (0)	
Pro		1.1 (1)					
Gly	0.4 (0)	3.5 (4)		1.1 (1)	0.4 (0)	0.6[e] (0)	1.0 (1)
Ala		1.0 (1)		1.0 (1)	0.3 (0)		
Cys				0.6[d] (1)			
Val	1.0 (1)	2.0[c] (3)					
Met							(1)[f]
Ile				0.9 (1)			
Leu	1.0 (1)	1.0 (1)	1.0 (1)				
Tyr					1.0 (1)	1.0 (1)	
Phe				1.0 (1)	1.0 (1)		
His							
Lys	0.5[c] (1)						
Trp							
Arg		1.0 (1)	1.1 (1)				
total	4	14	2	7	5	3	2
residue no.	1–4	5–18	19–20	21–27	28–32	30–32	33–34

[a] Values listed as residues per mole. Numbers in parentheses are the assumed integral values. [b] Experimental values not corrected for destruction or for incomplete hydrolysis. [c] Partially destroyed by ninhydrin. [d] Detected as aminoethylcysteine, value not corrected. [e] Contamination from C2 which runs close in paper chromatography. [f] Qualitatively detected as homoserine.

isolated and characterized tryptic peptides covering the whole V_H region to complete and confirm the automatic sequence data. Automatic peptide sequencing and peptide overlaps were used to complete the sequence determination in the regions which were not established by sequencing of the large fragments and to verify the assignments made at the end cycles of the sequencer runs (see Figure 1).

H chain Hil has pyrrolidonecarboxylic acid as N-terminal residue and was therefore inaccessible to the coupling reagent of Edman degradation. This fact has traditionally been a major difficulty for N-terminal sequencing of blocked proteins. Previous attempts to make accessible the N terminus of these proteins by chemical (Takahashi & Cohen, 1969) or enzymatic (Doolittle & Armentrout, 1968; Szewczuk & Mulczyk, 1969) procedures have proven only partially satisfactory. Recently, a technique has been proposed (Podell & Abraham, 1978) which uses calf liver pyroglutamate aminopeptidase commercially available as deblocking agent. We have successfully applied this procedure to the deblocking of both H chain Hil and its N-terminal piece (residues 1–99), which enabled us to automatically sequence the N-terminal portion of the molecule.

It has been claimed that polybrene increases significantly the retention of hydrophobic peptides in the spinning cup of the sequencer, thus improving the automatic sequencing capabilities of these peptides (Tarr et al., 1978; Klapper et al., 1978). We have used polybrene in order to sequence an unusually long tryptic peptide (T17, see Figure 1), which extends from positions 99 to 125, including the end of V_H and the beginning of C_H1. This is an insoluble peptide with a large content of nonpolar residues (see Table I). Only in the presence of polybrene were we able to extend the automatic sequencing to the C terminus (see Figure 4D). This fact adds to evidence previously obtained in other laboratories (Tarr et al., 1978; Klapper et al., 1978) in support of the view that polybrene is useful in the automatic liquid-phase sequencing of some peptides. However, we did not observe any significant improvement in sequencing the whole protein or other large fragments.

A comparison of the amino acid sequence of V_H Hil with other human V_H region sequences shows that V_H Hil belongs to the V_HIII subgroup of human immunoglobulins. A detailed comparison with protein Nie, a typical representative of this subgroup (Ponstingl et al., 1970), shows that 92 out of 121 residues are identical in both proteins (76%). Of the remaining 29 residues, 20 can be accommodated by a single-base change. In this comparison two gaps (positions 105 and 107) were introduced in protein Nie to maximize homology. As expected, most of the nonhomologous residues belong to the hypervariable regions. Capra et al. (1973) proposed that lysine at position 3 of the V_HIII group is specific for mouse and seal, where the other species examined have glutamine. We have found lysine at this position for human IgG Hil. Therefore, position 3 may not be "species specific". V_H Hil differs markedly from V_H New (Poljak et al., 1977) which belongs to subgroup V_HII of human immunoglobulins. A comparison of both V_H regions shows that only 56 out of 121 positions are identical in both proteins (46%). Four gaps, at positions 62, 101, 103, and 104, in V_H New are necessary to maximize homology. Saul et al. (1978) have reported that in V_H New residues 37, 39, 43–47, 49, 58–61, 95, 99–100, 105–106, and 108–114 (numbering changed to include the gaps introduced here) are involved in intersubunit (V_H–V_L) contacts. The core of the contacting region determined by Val-37, Gln-39, Leu-45, Tyr-95, and Trp-111 is conserved in V_H Hil. Other residues such as Asp-Asp-Thr (59–61), Leu (100), and Gly (106) in V_H New which make contact with hypervariable or variable residues of the L chain are replaced by Thr-Tyr-Tyr (59–61), Pro (100), and Phe (106) in V_H Hil. Residues 59–61, 100, and 106 in V_H New are in contact with residues Ser-Leu-Arg (93–95), Arg (95), and Lys (33) in V_L New, respectively (Saul et al., 1978). These positions in V_L Hil are replaced by Ser-Ala-Ser (93–95), Ser (95), and Tyr (33) (Lõpez de Castro et al., 1978). The significance of these "nonconserved" intersubunit contacts has been discussed by Saul et al. (1978).

In addition, most of the nonpolar amino acid side chains placed between the two β sheets which make up the backbone of the V_H sequences are invariant or chemically analogous in both proteins. In V_H Hil, these are: Leu-4, Leu-18, Leu-20, Cys-22, Met-34, Trp-36, Val-48, Phe-68, Ile-70, Leu-79, Met-81, Met-83, Leu-86, Ala-92, Val-94, Cys-96, Tyr-110, Val-115, Val-117, and Val-119. Examination of the three-dimensional structure of Fab New clearly shows that the sequence Val-Ser-Ser (residues 119–121) constitutes the end of the V_H region. After a sharp bend, Ala-122 is at the beginning of the C_H1 homology region (Poljak, 1975).

The sequence at the heavy chain hypervariable regions of both proteins (Hil and New) is significantly different regarding either identity or chemical analogy of residues at analogous positions. In addition, the second and fourth hypervariable regions of V_H Hil possess one and three additional residues, respectively (residues 62, 101, 103, and 104), as compared with V_H New. These differences, as well as the differences discussed before between the hypervariable regions of the light chains (Lõpez de Castro et al., 1978), suggest that proteins New and Hil may differ significantly in the nature and conformation of their combining sites. A quantitative comparison will be made possible when a high-resolution electron density map of Fab Hil becomes available.

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Structural and Antigenic Studies of an Idiotype-Bearing Murine Antibody to the Arsonate Hapten†

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ABSTRACT: Mice of strain A/J responded to repeated intraperitoneal injection of *Limulus* hemocyanin derivatized with arsanilic acid by producing large quantities (approximately 5 mg/mL of ascites fluid) of IgG antibodies specific for this hapten. The antibodies possessed a characteristic idiotypic determinant and exhibited restricted heterogeneity as demonstrated by isoelectric focusing and primary N-terminal amino acid sequence analysis of isolated light and heavy polypeptide chains. Both light- and heavy-chain sequences were comparable to those of myeloma proteins in lack of heterogeneity. The N terminus of the light chain exhibited $V_{\kappa I}$ sequence and only one position in the first 30 residues showed more than one amino acid. No variability was observed in the first 10 N-terminal residues of the heavy chain. Rabbit antiserum to the idiotype blocked binding of hapten by the purified antibody. The presence of both light- and heavy-chain antigenic determinants was required for optimal formation of the idiotypic determinant.

It is possible, using certain mammals and immunization protocols, to raise antibodies of restricted structural heterogeneity (Appella et al., 1973; Osterland et al., 1966). Moreover, antibodies can be produced which react with the variable region combining site for antigen on the original antibodies (Capra & Kehoe, 1975). The anti-antibodies, or antiidiotypic antibodies, are useful probes for determination of the presence of antibody-like receptor molecules on the surface of lymphocytes (Binz & Wigzell, 1977; McKearn, 1974; Rajewsky & Eichmann, 1977). In this study, we induce antibodies to the arsonate hapten in strain A/J mice by a modification of the procedure of Tung & Nisonoff (1975). We report that the purified antibody was predominantly of the IgG 2a class and showed restricted heterogeneity as defined by isoelectric focusing and amino acid sequence analysis of the constituent polypeptide chains. The intact molecule exhibited idiotypic determinants, as determined by the use of rabbit antibodies produced against the $(Fab')_2$ fragment of the mouse antibody. Presence of both light- and heavy-chain determinants was required for optimal formation of the idiotypic determinant.

Materials and Methods

Antigens and Affinity Reagents. *p*-Arsanilic acid (ARS)[1] (Eastman Kodak, Rochester) was diazotized and coupled to bovine serum albumin (BSA) (crystalline, Grand Island Biological Co., New York) or *Limulus* hemocyanin (Hcyn) (prepared from hemolymph by zone electrophoresis as previously described by Marchalonis & Edelman, 1968). The arsenic content of the derivatized proteins was determined (Australian Microanalytical Service, Chemistry Department, University of Melbourne), and the molar ratios of hapten to protein were calculated as 10 mol of ARS/mol of BSA and 8 mol of ARS/Hcyn subunit of 60 000 daltons. The ARS_{10}-BSA was coupled to Sepharose 4B in a CNBr-catalyzed reaction (Haustein & Warr, 1976). The solid-phase affinity reagent contained 5 mg of derivatized BSA/mL of gel. Elution buffers consisted of pH 2.2 glycine hydrochloride (0.05 M) which was 0.15 M in NaCl and 3.5 M NaSCN (in glass-distilled H_2O).

Immunization. A/J mice maintained at the Walter and Eliza Hall Institute animal facility were immunized according to the procedure of Tung & Nisonoff (1975), with the only exception that *Limulus* Hcyn, rather than keyhole limpet Hcyn, was the carrier to which arsanilic acid was coupled. Ascites fluids were tapped after 4 weeks. A total of 24 mice were used.

Purification of Antibody to the ARS Hapten. Clarified ascites fluid was passed through the ARS_{10}-BSA immunoadsorbent. Antibody was eluted using glycine hydrochloride buffer, pH 2.2, followed by 3.5 M NaSCN.

Preparation of Polypeptide Chains. Purified antibody was reduced with 2-mercaptoethanol and alkylated with iodoacetamide, and chains were separated by gel filtration on Sephadex G-200 (Pharmacia, Uppsala) in 1 M propionic acid/4 M urea as described by Edelman & Marchalonis (1967).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in sodium dodecyl sulfate ($NaDodSO_4$)-containing buffers as described by Laemmli & Favre (1973).

Isoelectric Focusing. This was carried out using the LKB Multiphor flat-bed electrophoresis apparatus. Two-millimeter

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[1] Abbreviations used: ARS, *p*-arsanilic acid; BSA, bovine serum albumin; Hcyn, hemocyanin; NaCl-*P*, phosphate-buffered saline; RIA, radioimmunoassay; $NaDodSO_4$, sodium dodecyl sulfate.

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