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Homogeneous Rabbit Immunoglobulin Lacking Group a Allotypes: Amino Acid Sequence Analysis of the Heavy Chain†

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ABSTRACT: The partial amino acid sequence of rabbit a-negative heavy chain has been determined for residues 1–43 as: <EEQLEESGGGLVQPGGSLKLSCKGSGFDFSVY-GVTWVRQAPGK; and for residues 64–120 as: MNG-RFTISSDNAQNRLYLQLNSLTAADTATYFCARSMV-VVAGVHSYFDVWGPGTLVTV. Comparison of this sequence with the human heavy chain subgroup III shows homology of 78% suggesting that a common ancestral variable region gene existed in mammals prior to speciation. The constant region of the a-negative chain is structurally identical with that of a-positive chains, whereas the variable region differs substantially between a-positive and a-negative molecules. These findings support the concept that two genes encode one immunoglobulin polypeptide chain and demonstrate the existence in the rabbit of variable region subgroups similar to those reported for humans and other species. A novel approach to the initial fragmentation of the heavy chain was developed in this study. This method, which involved digestion of the H chain with the protease V8, produced a free N terminus and should have wide application in future studies on heavy chains with blocked amino terminals.

Rabbit immunoglobulin heavy (H[1]) chain V regions carry several genetically determined antigenic markers (Oudin, 1960a,b), analyses of which have provided insight into basic immunogenetic questions. Although the majority of rabbit V_H regions carry group a allotypes, molecules lacking these specificities were revealed by quantitative determination of the group a markers in immunoglobulin pools (Dray et al., 1963), by homozygous allotype suppression experiments (David & Todd, 1969), and by the production of homogeneous antibodies that do not react with antisera directed against the group a specificities (Kindt et al., 1970). Kim & Dray (1972) have shown that there are allotypic markers other than those of group a present on this minority population.

Amino acid sequence studies on the H chains from immunoglobulin pools (Wilkinson, 1969; Mole et al., 1971; Johnstone & Mole, 1977) and from homogeneous antibodies (Fleischman, 1971; Jaton, 1975; Margolies et al., 1977) have demonstrated differences between the three group a allotypes, a1, a2, and a3. Structural differences between molecules possessing the group a allotypes and those lacking them were indicated by compositional differences and by sequence analysis of N-terminal peptides (Prahl et al., 1973; Tack et al., 1973). More recently, the sequence of a-negative molecules in an immunoglobulin pool was found to be 80% homologous to the human V_HIII subgroup in the N-terminal 20 residues (Johnstone & Mole, 1977).

A homogeneous immunoglobulin lacking group a allotypes was produced by rabbit 3547 upon hyperimmunization with group A streptococci. The L chain of this immunoglobulin was of the b4 allotype and its V region structure has been determined (Thunberg & Kindt, 1976). The present study, concerning sequence analysis on the a-negative H chain of this immunoglobulin, was initiated to investigate the structural basis of V region serological markers and to determine whether structurally identical C regions are associated with both a-positive and a-negative V regions (Dreyer & Bennett, 1965). Preliminary accounts of these findings have been presented

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[1] Abbreviations used: H chain, heavy chain; L chain, light chain; V region, variable region; C region, constant region; V_HI, V_HII, V_HIII, human heavy chain variable region subgroups I, II, and III, respectively; CmCys, *S*-carboxymethylcysteine; Dnp-, 2,4-dinitrophenyl-; CN, V8, T, TL, peptides from CNBr, V8-protease, tryptic and thermolytic cleavage, respectively; SPITC, 4-sulfophenyl isothiocyanate.

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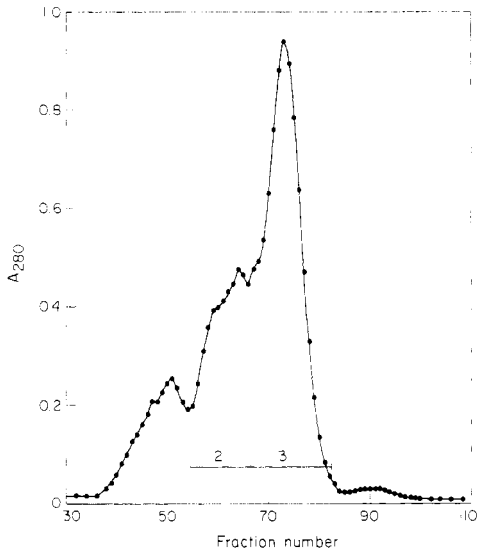

FIGURE 1: Gel filtration of the CNBr cleavage products of 3547 H chain in a column (3.0 × 140 cm) of Sephadex G-100 in 0.2 M sodium formate, pH 3.3, containing 6 M urea. Fraction size was 10 mL. (●—●) A_{280}.

(Fraser et al., 1977; Johnstone & Thunberg, 1977).

Materials and Methods

Enzymes. Trypsin was treated before use with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Kostka & Carpenter, 1964). The "V8-protease" enzyme from *Staphylococcus aureus,* strain V8 (Houmard & Drapeau, 1972), which cleaves peptide bonds on the carboxyl side of glutamic acid residues was purchased from Miles Laboratories, Inc.

Chemicals. Pyridine was refluxed over ninhydrin (2 g/L) for 1 h and then redistilled. Formic acid was redistilled before use. Iodoacetic acid was recrystallized from diethyl ether and stored in the dark at 4 °C. Iodoacetic-*2*-^{14}C (48 mCi/mmol) was obtained from Amersham/Searle and stored at −20 °C as a 1 mCi/mL solution in water.

Isolation of 3547 H Chains. A homogeneous immunoglobulin was isolated from the serum of rabbit 3547, which had been hyperimmunized with group A streptococci, and its constituent chains separated, after reduction and carboxamidomethylation, as described by Thunberg & Kindt (1976). The immunoglobulin was shown to be a-negative by lack of reaction with insolubilized anti-a-allotype antisera in a radioimmunoassay (Gottlieb et al., 1975).

CNBr Cleavage of 3547 H Chains. Cleavage of the H chains with CNBr and fractionation of the products were carried out as described by Fruchter et al. (1970).

V8-Protease Digestion of 3547 H Chains. The H chains (70 mg) were completely reduced and ^{14}C-carboxymethylated (O'Donnell et al., 1970) in 7 M guanidine hydrochloride, 0.5 M Tris-HCl, pH 8.2 (20 mg of protein/mL), and then citraconylated (Mole et al., 1971) to keep the unfolded chains in solution upon removal of the guanidine. Excess reagents were removed by dialysis against 0.5% NH_4HCO_3, pH 8.0, and the chains were digested with V8 protease (2% w/w) at 37 °C for 15 h in this buffer. The digestion was terminated by addition of an equal volume of formic acid and the mixture applied directly to a column (2.2 × 190 cm) of Sephadex G-100 in 50% (v/v) formic acid.

Tryptic Digestion of Fragment V8-1. The V8-protease fragment V8-1 was citraconylated (to block the ε-amino group of lysine residues) and digested with trypsin as described by Mole et al. (1971) for the CNBr fragment of heavy chains, C-1; the citraconylation was carried out in 4 M guanidine hydrochloride to help solubilize the fragment. After removal of the blocking groups (Mole et al., 1971), the tryptic peptides were fractionated on a column (1.8 × 190 cm) of Sephadex G-50 fine in 50% (v/v) formic acid.

Enzymatic Digestion of Peptides. The conditions used were as follows: tryptic digestion in 1% NH_4HCO_3, pH 8.2, at 37 °C for peptide CN32 (150 μg of enzyme/μmol of peptide for 2 h), and for peptide CN22 (300 μg of enzyme/μmol of peptide for 1.5 h); thermolytic digestion of peptide V8-6A in 0.1 M ammonium acetate, pH 8.5, containing 5 mM $CaCl_2$ (600 μg of enzyme/μmol of peptide) and of peptide CN35 in 0.5% NH_4HCO_3, pH 8.0 (100 μg of enzyme/μmol of peptide) at 37 °C for 3 h; V8-protease digestion in 0.5% NH_4HCO_3, pH 8.0, for peptide CN31-T1 (70 μg of enzyme/μmol of peptide) for 15 h at 37 °C.

Fractionation of Peptides. High voltage paper electrophoresis was carried out using the apparatus and buffers described by Chen & Krause (1975). The charge of a peptide was determined from its mobility at pH 6.5 (Offord, 1966).

Ion-exchange chromatography was performed using a column (0.7 × 6.0 cm) of DEAE-Sephadex (A-25) or SP-Sephadex (C-25) (Pharmacia Fine Chemicals). A concave gradient (reservoirs of 50 mL and 25 mL) from 0.01 to 0.5 M NH_4HCO_3, pH 8.5, or from 0.05 M pyridine formate, pH 3.0, to 1 M pyridine formate, pH 5.0, was used to elute the anion and cation exchanges, respectively. In some cases, the buffers were made 6 M in urea which had been freshly deionized on a mixed bed resin; urea was removed from the peptides after separation by desalting on a column (1.2 × 32 cm) of Sephadex G-25 medium in 1 N NH_4OH. Peptides were detected in the eluate by radioactivity, absorption at 230 and 280 nm, in NH_4HCO_3 buffers, or by reaction with fluorescamine (Roche), in pyridine formate buffers.

Analytical Methods. Details of amino acid analysis and sequence determination from the C terminus by digestion with carboxypeptidases A and B have been described previously (Johnstone & Kindt, 1977). Characterization of ^{14}C-carboxymethylcysteine-containing peptides in column eluates was carried out by tryptic–chymotryptic digestion followed by paper electrophoresis and autoradiography (O'Donnell et al., 1970). The dansyl–Edman procedure, combined with identification of the phenylthiohydantoin-amino acids released, was performed as detailed by Johnstone and Mole (1977). Automated sequence determination of peptides was carried out using a Beckman 890-B sequencer with a peptide program (111374) as described previously (Thunberg & Kindt, 1976). Tryptic peptides with C-terminal lysine were treated with SPITC (4-sulfophenyl isothiocyanate) to minimize loss during automated sequencing (Inman et al., 1972).

Numbering of Residues. The numbering system in the present report for the V region is taken from the human V_HIII sequence (Capra & Kehoe, 1975). Cysteines 96, 149, and 204 correspond to cysteines 92, 146, and 201 in previously reported rabbit H chain studies (O'Donnell et al., 1970; Mole et al., 1971; Pratt & Mole, 1975).

Results

CNBr Cleavage of 3547 H Chains. Fractionation on Sephadex G-100 of the CNBr peptides of 3547 H chains (Figure 1) gave a profile different from that usually obtained for rabbit H chains (Fruchter et al., 1970). The large C-1 peptide, consisting of the N-terminal half of the chain, was not observed (elution position, fraction 55) and more low molecular weight material was obtained.

After complete reduction and ^{14}C-carboxymethylation

TABLE I: CNBr Peptides of 3547 H Chain.[a]

Amino acid	H chain	Peptides CN32	CN35	CN22	CN31	CN36	CN34	CN42
Cys[b]	8.5							
Cm-Cys		1.1	1.1	4.8	1.5	0.9	0.9	
Asp	34.0	5.7	4.3	7.9	7.0	0.1	6.2	1.1
Thr	41.2	3.9	3.8	17.2	7.2	1.1	3.2	1.1
Ser	46.7	5.6	3.3	17.2	4.9	3.0	6.2	2.8
Glu	43.7	6.4	2.5	5.6	12.1	2.0	6.2	2.2
Pro	38.7	2.5		16.7	8.9	2.2	2.5	1.2
Gly	34.0	7.6	1.7	12.3	3.2	1.3	6.7	1.2
Ala	22.5	4.2	3.5	6.8	5.0	0.1	2.2	1.1
Val	46.8	3.8	0.4	21.2	9.6	1.1	5.0	
Ile	14.0	2.7	1.0	1.2	4.9		2.2	1.0
Leu	28.9	5.2	4.1	10.8	4.8	2.0	3.8	1.0
Tyr	14.0	3.3	1.9	3.1	2.0		3.8	0.8
Phe	15.6	3.2	1.9	5.2	3.0		3.1	
His	7.7	0.7		2.1	1.8			3.4
Lys	25.6	2.7		7.1	5.8		3.5	1.0
Arg	18.0	1.8	2.7	2.3	5.7	1.9	1.0	1.0
Hse[c]		0.3	0.5	0.4	0.3	0.4	0.4	
Met[b]	5.9							
No. of residues		64	36	152	99	17	60	18
Residue position		1-64	65-100	101-252	253-351	352-368	369-428	429-446
Residues[d] identified				101-131	253-279	352-363	369-392	

[a] Compositions are given in residues per molecule. [b] Cysteine and methionine were estimated as cysteic acid and methionine sulfone after performic acid oxidation of H chains. [c] Homoserine lactone was not converted to homoserine prior to these analyses. [d] Residues identified by automated sequence analysis.

TABLE II: Amino-Terminal Sequence Analysis of 3547 C Region Peptides.[a]

Peptide	Residue positions	
CN22	101-252	VVAGVHSYFDVWGPGTLVTVSSGQPKAPSVF
CN22-T-C[b]	127-152	(A)PSVFPLAPCCGDTPSSXVXLGCLV(K)
CN22-T-D1	153-173	GYLPEPVTV
CN22-T-H	174-179	TFPSVR
CN22-T-B	180-214	QSSGLYSLSSVVSV
CN22-T-G	215-217	VDK
CN22-T-E	218-248	TVAPSTCSKPTCPPPELLGGPSV
CN22-T-F	249-252	DTLHse
CN31	253-351	ISXTPEVTCVVVDVSQDDPZVZFTXYI
CN36	352-368	GPXREELSSXSV
CN34	369-428	INGFYPSDISVEWEKNGKAEDNYK

[a] Sequence analysis carried out using automated sequencer with exception of peptides CN22-T-H, CN22-T-G, and CN22-T-F. [b] Treated with SPITC prior to sequence analysis causing loss of N-terminal residue (Ala) and C-terminal lysine.

(O'Donnell et al., 1970), fractionation of pool 3 (Figure 1) on the same Sephadex G-100 column in 0.2 M sodium formate, pH 3.3, containing 6 M urea, yielded all of the normal C region CNBr peptides spanning residues 253 to 428 (Tables I and II). The small peak following pool 3 (Figure 1) yielded a peptide (CN42) with composition characteristic of the C-terminal octadecapeptide (Hill et al., 1967) which spans residues 429–446. The amino acid compositions and N-terminal sequences of these peptides (Tables I and II) were entirely consistent with the C region structure of a-positive chains (Hill et al., 1967; Fruchter et al., 1970; B. A. Fraser, D. G. Klapper, & A. L. Thunberg, unpublished).

Three additional CNBr peptides (Table I) were isolated from pools 2 and 3 (Figure 1); these spanned residues 1–252 and indicated the presence of 2 methionine residues in the V region, thus explaining the unusual CNBr cleavage pattern. Peptides CN32 and CN35 were resolved in insufficient amounts for complete characterization; some structural data were obtained by tryptic and thermolytic digestion of these peptides (Table III and Figures 5 and 6).

Automated sequence analysis of peptide CN22 showed that it was the product of cleavage at a methionine residue in the third hypervariable region and contained the N-terminal portion of the C region (Table I, Table II, and Figure 6). A tryptic digest of this peptide (350 nmol) was fractionated on a column (1.8 × 180 cm) of Sephadex G-50 Fine in 0.1 M NH_4OH and the resultant peptides purified by cation-exchange chromatography (peptide CN22-T-B) or paper electrophoresis (Table IV). Characterization of these peptides (Tables II and IV) allowed the sequence of peptide CN22 to be almost completely assigned. The peptides (Table II) were aligned by homology with Pratt & Mole (1975) and on the basis of the N-terminal sequencer runs. All results were consistent with the structure of a-positive chains of the d12 allotype in this region (Prahl et al., 1969; Pratt & Mole, 1975).

V8-Protease Digestion of 3547 H Chains. Digestion of 3547 H chains with V8-protease gave the elution profile shown in Figure 2. Characterization of [^{14}C]carboxymethylcysteine residues in the eluate (O'Donnell et al., 1970) detected only cysteines 22, 96, 149, and 204 in pools 1 and 2, cysteines 321

TABLE III: Peptides Derived from CNBr Fragments CN32 and CN35.[a]

Amino acid	Tryptic peptides			V8-protease peptides		Thermolytic peptides		
	CN32-T1	CN32-T5	CN32-T6	CN32-T1 V8-1	CN32-T1 V8-2	CN35-TL4	CN35-TL1	CN35-TL5
Cm-Cys		0.8					0.7	
Asp	0.1	0.2				1.3		
Thr	0.1					2.7		
Ser	1.9	1.5	0.1	0.2	1.8	0.3	0.2	1.1
Glu	5.2		1.0	5.7	1.1			
Pro	1.9		1.2		1.0			
Gly	4.5	0.5	1.0	0.1	4.7		0.1	0.2
Ala	0.3	0.3	1.0			2.8		1.0
Val	0.8				1.0			
Ile						0.2		
Leu	3.0	1.0		1.0	1.8	1.0	0.2	
Tyr						1.0		
Phe							1.0	
Lys	0.9	1.0	1.0		1.0			
Arg								0.9
Hse								0.9
Mobility[b]								
pH 3.5		+0.38	+0.61					
pH 6.5	−0.39	0	+0.52	−1.0	+0.32	−0.22	−0.57	+0.58

[a] Compositions are given in residues per molecule. [b] Mobilities are expressed relative to ϵ-Dnp-lysine (0), aspartic acid (−1.0), and lysine (+1.0).

TABLE IV: Tryptic Peptides of Fragment CN-22.[a]

Amino acid	Peptide						
	CN22-T-C	CN22-T-D1	CN22-T-H	CN22-T-B	CN22-T-G	CN22-T-E	CN22-T-F
Cm-Cys	1.8	0.2		0.1		1.3	
Asp	1.5	1.8		2.1	1.0		1.0
Thr	3.6	3.5	1.0	3.9		2.8	1.0
Ser	2.7	1.1	0.9	7.6	0.2	2.6	0.4
Glu	0.4	1.0		2.1		1.1	
Pro	3.7	1.7	1.0	1.6		8.0	
Gly	2.5	2.5		1.3	0.2	2.0	0.3
Ala	1.9	0.2		2.2		1.1	0.2
Val	3.7	3.0	1.1	4.8	1.0	2.0	
Ile						1.1	
Leu	3.1	2.0		2.0		2.0	1.0
Tyr	0.4	1.1		0.7			
Phe	1.2		1.1			2.2	
His				0.9			
Lys	0.6			1.2	1.0	2.4	
Arg		1.3	1.1				
Hse							0.7
Mobility[b]							
pH 3.5			+0.52		+0.52		
pH 6.5	−0.13	−0.23				+0.28	−0.46
Residue position	127–152	153–173	174–179	180–214	215–217	218–248	249–252
Residues identified							
Automated[c]	127–151	153–161		180–193		218–240	
Manual[d]			174–179		215–217		249–252

[a] Compositions are given as residues per molecule. [b] Mobilities are expressed as in Table III. [c] Residues identified by N-terminal automated sequence analysis. [d] Residues identified by the dansyl–Edman procedure.

and 367 in fractions 76–83, and cysteines 261 and 425 in fractions 81–90. The cysteine-22 peptide was more basic than in a-positive chains (mobility at pH 3.5 + 0.34 relative to ϵ-Dnp-lysine, 0, and lysine, +1.0) because of the lysine for threonine interchange at position 23 (Figure 5). The amino acid compositions of pools 1 and 2 were nearly identical (Table V) and the dansyl reaction gave the N terminus of both as serine. It was concluded that these two pools contained the same fragment (spanning residues 7–258), material in pool 1 being aggregated; this fragment was termed V8-1 and, because it contained most of the V region, it was investigated further (see below).

The later eluting fractions (70–105) consisted entirely of smaller C region peptides except for the blocked N-terminal hexapeptide which was purified from fractions 88–95 by paper electrophoresis at pH 6.5 (peptide V8-6A; Table V) and is identical with CN32-T1-V8-1 (Table III). Thermolytic digestion of this peptide gave two peptides (Table V) in approximately 60% yield after one paper electrophoresis step; the complete sequence of peptide V8-6A-TL-1 was inferred (Table V and Figure 5). The other product (V8-6A-TL-2) had no free amino group, contained only glutamic acid and its mobility at pH 3.5 and 6.5 was identical with that of <Glu-Glu-Gln, the N-terminal peptide of rabbit a-negative chains (Wilkinson,

TABLE V: Variable Region Peptides Obtained from a V8-Protease Digest of 3547 H Chain.[a]

Amino acid	V8-protease peptides V8-1	V8-2	V8-6A	Thermolytic peptides V8-6A-TL2	V8-6A-TL1
Cm-Cys	6.9	6.4			
Asp	19.9	22.3			
Thr	26.9	25.9			
Ser	31.3	30.5	0.1		
Glu	12.1	12.6	5.0	3.0	2.2
Pro	16.7	14.2			
Gly	25.7	25.7	0.1		
Ala	14.8	14.5			
Val	24.3	24.9			
Met	12.2	2.1			
Ile	6.0	6.0			
Leu	20.5	19.2	1.0		1.0
Tyr	7.3	6.4			
Phe	9.1	9.0			
His	2.3	3.4			
Lys	9.9	10.4			
Arg	6.6	6.2			
Residue position	7-258	7-258	1-6	1-3	4-6
Mobility[b]					
pH 3.5			−0.43	−0.50	+0.10
pH 6.5			−0.91	−0.88	−0.73

[a] Compositions are given as residues per mole. [b] Mobilities are expressed relative to ε-Dnp-lysine (0), lysine (+1.0), and pyrrolid-2-one-5-carboxylic acid (−1.0).

1969; Prahl et al., 1973; Johnstone & Mole, 1977). Pronase digestion of this blocked peptide (2 μg of enzyme/nmol of peptide at 37 °C for 16 h in 1% NH_4HCO_3, pH 8.2) gave two products. Electrophoresis of the digest at pH 6.5 revealed two peptides in addition to undigested material: one was neutral and ninhydrin positive; the other was ninhydrin negative and had a mobility of −1.15 (relative to ε-Dnp-lysine, 0, and aspartic acid, −1.0). All of these data support the structure <Glu-Glu-Gln for the N terminus of 3547 H chains.

CNBr Cleavage of Fragment V8-1. Fractionation of the products of CNBr cleavage of fragment V8-1 (20 mg/mL in 70% formic acid at a protein:CNBr ratio of 1:5 for 24 h at 4 °C) on a column (1.8 × 190 cm) of Sephadex G-50 Fine in 50% (v/v) formic acid gave the profile shown in Figure 3. Cysteines 149 and 204 were detected in fractions 36–42 and cysteines 22 and 96 in fractions 40–45. The double peak (fractions 48–59) contained cysteine-22 in the early fractions and cysteine-96 in the later fractions.

Dansylation of the first peak gave a major Dns-Val-Val spot and it was concluded that this pool contained the peptide spanning residues 101–252 which had been characterized previously (peptide CN22, Tables I, II, and IV, Figure 6). A large peptide containing cysteine-22 and -96 was present in the second peak; this was presumably the result of incomplete cleavage at methionine-64. However, because of its low yield (30% of the total cysteine-22- and 96-containing peptides) and contamination with peptide CN22, it was not investigated further.

The partially resolved V region peptides (fractions 48–59) were purified by cation-exchange chromatography in urea (Table VI). Peptide V8-1-CNC-1 was completely characterized and peptide V8-1-CNC2 was partially characterized by amino acid analysis, automated sequence analysis, and by digestion with carboxypeptidase A and B (Figures 5 and 6).

Amino acid analysis of the later fractions of Figure 3 detected a peptide (V8-1-CND; Table VI) eluting in fractions 72–80 and this was purified by paper electrophoresis at pH 3.5.

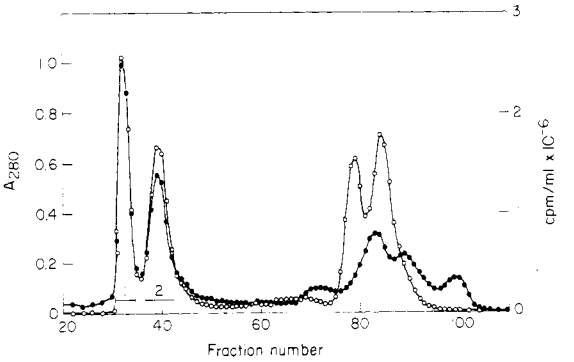

FIGURE 2: Gel filtration of V8-protease digest of completely reduced and [^{14}C]-carboxymethylated 3547 H chain on a column (2.2 × 190 cm) of Sephadex G-100 in 50% (v/v) formic acid. Fraction size was 7 mL. (●—●) A_{280}; (○—○) radioactivity (cpm/mL × 10^{-6}).

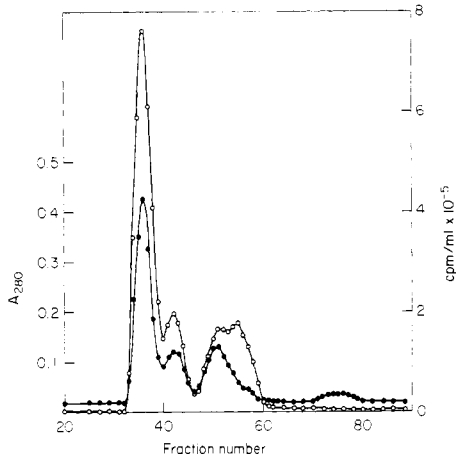

FIGURE 3: Gel filtration of the CNBr cleavage products of fragment V8-1 on a column (1.8 cm × 190 cm) of Sephadex G-50 fine in 50% (v/v) formic acid. Fraction size was 5.8 mL. (●—●) A_{280}; (○—○) radioactivity (cpm/mL × 10^{-5}).

By the dansyl-Edman procedure, the sequence of this peptide was determined as Ile-Ser-Arg-Thr-Pro-Glu; it was therefore derived from the C terminus of fragment V8-1 and agrees with the known a-positive C-region structure (residues 253–258).

Tryptic Digestion of Citraconylated Fragment V8-1. Figure 4 shows the fractionation of the products of cleavage at the arginine residues of fragment V8-1. The first peak (fractions 30–37) contained all four cysteines present in fragment V8-1 and was assumed to be aggregated or undigested material. Cysteines 149–204 were present throughout the second peak with cysteine-149 predominant in fraction 43 and cysteine-204 in fractions 40 and 47. Pool D1 contained cysteine-22 and -96 and pool E contained only cysteine-96.

The cysteine-149-containing peptide in pool B2 was purified by anion-exchange chromatography in urea (peptide V8-1-TB2; Table VI); its N-terminal sequence, determined by automated sequence analysis, overlapped the CNBr peptides V8-1-CNC-1 and CN22 (Figure 6).

Pool D1 (Figure 4) was further fractionated on a column (1.8 × 190 cm) of Sephadex G-50 Fine in 0.05 M NH_4OH and two peaks were obtained from which two radioactive peptides (V8-1-TD1-A and V8-1-TD1-B; Table VI) were isolated by anion-exchange chromatography in urea. These peptides were not completely pure (Table VI) but only one N-terminal sequence was detected by automated sequence analysis (Figure 5).

TABLE VI: CNBr and Tryptic Peptides of Fragment V8-1.[a]

Amino acid	CNBr peptides			Tryptic peptides						
	V8-1-CNC-2	V8-1-CNC-1	V8-1-CND	V8-1-TD1-B	V8-1-TD2	V8-1-TF	V8-1-TE	V8-1-TD1-A	V8-1-TB2	V8-1-TG
Cm-Cys	0.2	0.2		0.2			0.2	0.1	0.9	
Asp	4.1	5.2	0.1	1.9	2.9	2.9	2.3	5.1	4.6	
Thr	2.6	3.6	1.0	1.2	1.8	1.0	2.6	3.4	8.5	1.0
Ser	6.3	4.2	1.2	3.7	1.6	1.9	1.5	3.5	8.0	1.0
Glu	3.4	2.1	1.1	1.5	4.6	1.1	1.5	2.6	2.6	
Pro	4.1	1.1	1.4	0.7	2.1		0.6		6.9	1.3
Gly	11.0	2.2	0.2	7.5	1.0	0.4	1.4	3.4	9.3	0.1
Ala	3.1	4.4	0.1	1.0	2.5	1.0	3.6	4.2	3.5	
Val	4.2	0.7		3.2	1.9		0.4	0.9	10.6	1.0
Met					0.3				1.2	
Ile	2.5	1.1	1.0	0.8	0.8	1.0	0.6	1.3	0.6	
Leu	4.7	3.6		2.7	3.3		4.0	4.1	7.0	
Tyr	1.9	1.6		1.2	1.0		1.6	1.4	2.4	
Phe	2.8	1.9		1.8	1.6	0.9	1.0	1.7	1.9	0.9
His	0.3			0.1	0.9			0.6	1.1	
Lys	2.7	0.3		1.5	3.0		0.2	1.8	2.0	
Arg	1.8	2.7	0.8	0.9	1.2	1.2	1.0	1.0	1.0	1.0
Hse	0.5	0.9								
Residue position	7-64	65-100	253-258	7-38	39-67	68-78	79-98	68-98	99-173	174-179
Yield[b]	0.63	0.58	0.74	0.90	0.80	0.47	0.56	0.33	0.78	0.79
Mobility[c]										
pH 3.5			+0.50			+0.27				+0.53
pH 6.5			0			0				

[a] Compositions are given as residues per molecule. [b] Yields are expressed as mol of peptide per mol of fragment V8-1 after gel filtration. [c] Mobilities are expressed as in Table III.

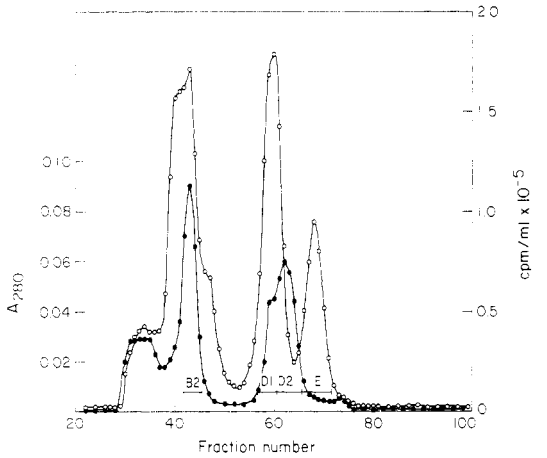

FIGURE 4: Gel filtration of a tryptic digest of citraconylated fragment V8-1 on a column (1.8 × 190 cm) of Sephadex G-50 fine in 50% (v/v) formic acid. Fraction size was 5.8 mL. (●—●) A_{280}; (○—○) radioactivity (cpm/mL × 10^{-5}).

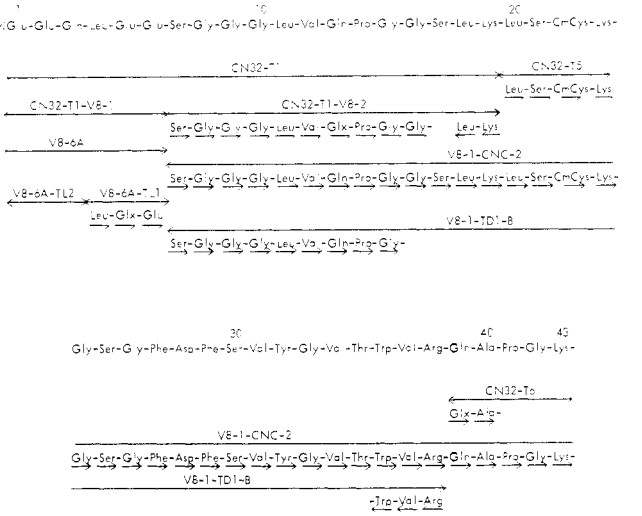

FIGURE 5: Amino acid sequence of residues 1-43 of 3547 H chain. Sequence determination from the N-terminal end was by automated sequence analysis, indicated by —►, or by the dansyl-Edman procedure, indicated by —→, and from the C-terminal end by hydrolisis with carboxypeptidase B, indicated by ←—, or with carboxypeptidase A and B, indicated by ←—. Identification of the dansyl derivative without hydrolysis is indicated by +►.

The unlabeled peptide in pool D2 (Figure 4) was purified by cation-exchange chromatography (V8-1-TD2; Table VI); no free N terminus was detected by dansylation or automated sequence analysis of this peptide. It was assumed to span residues 39-67 and to be blocked by cyclization of its N-terminal glutamine residues (Figure 5) during its isolation under acidic conditions; however, insufficient material remained for further characterization.

The cysteine-96-containing peptide V8-1-TE (Table VI) was purified from pool E (Figure 4) by anion-exchange chromatography and partially characterized by the dansyl-Edman procedure and digestion with carboxypeptidases A and B (Figure 6). Two peptides were detected in later fractions of Figure 4 by amino acid analysis (peptide V8-1-TF in fractions 73-79 and peptide V8-1-TG in fractions 77-83) and were purified by paper electrophoresis (Table VI). Characterization of these peptides showed that peptide V8-1-TG was identical with the C region peptide CN22-T-H (Tables II and IV) spanning residues 174-179, and placed peptide V8-1-TF at residues 68-78 (Figure 6).

Discussion

Rabbit H chains lacking group a allotypes comprise a small percentage of the normal IgG pool (5-30%); this figure is higher (65%) in IgA from animals of the a3 allotype (Johnstone & Mole, 1977). Sufficient amounts of these a-negative H chains have been obtained for structural studies by homo-

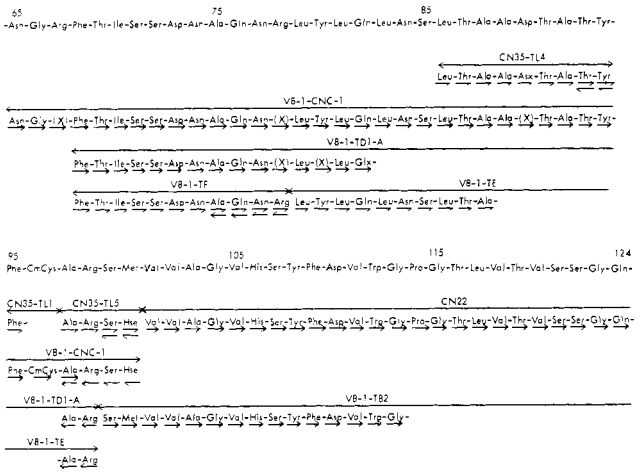

FIGURE 6: Amino acid sequence of residues 65–124 of 3547 H chain. Sequence determination from the N-terminal end was by automated sequence analysis, indicated by →, or by the dansyl–Edman procedure, indicated by →, and from the C-terminal end by hydrolysis with carboxypeptidase A, indicated by ←, or with carboxypeptidase B, indicated by ←, or with carboxypeptidase A and B, indicated by ←. X indicates unidentified residue on automated sequence analysis. ± indicates identification of the dansyl derivative without hydrolysis. Serine, glutamine, and asparagine could not be distinguished on amino acid analysis of carboxypeptidase digest.

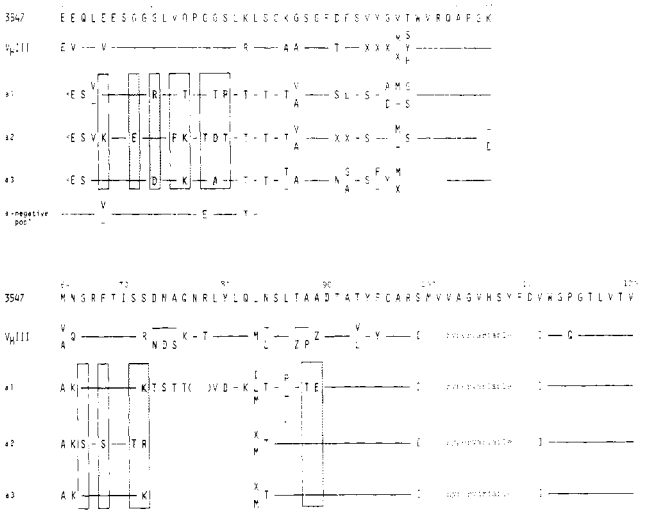

FIGURE 7: Comparison of the H chain V region sequences of 3547, human V_HIII, rabbit a1, a2, a3 and pooled a-negative immunoglobulins. Amino acid residues are listed by the single letter code. Line indicates identity to 3547 sequence. X represents residues unidentified or hypervariable. The parentheses (77–78) indicate deletion used to maximize homology. The human sequence is from Capra & Kehoe (1975) and the a1, a2, a3, and pooled a-negative sequences are from Wilkinson (1969), Mole et al. (1971), Fleischman (1971), Jaton (1975), Margolies et al. (1977), and Johnstone & Mole (1977). The stippled residues are thought to be related to the a allotype.

zygous allotype suppression (David & Todd, 1969) by the occasional production of a-negative homogeneous antibodies (Kindt et al., 1970) and from IgA pools (Johnstone & Mole, 1977). The material used in this study was obtained from a rabbit immunized with group A streptococcal vaccine. The IgG from preimmune serum of this rabbit showed normal levels of the group a allotypes a2 and a3.

The amino acid composition of 3547 H chain (Table I) differs from that of a-positive chains notably in the lower threonine and higher glutamic acid content. Similar compositional differences have been observed for a-negative molecules obtained in homozygous allotype suppression experiments (Tack et al., 1973). In spite of these compositional differences between 3547 H chain and a-positive molecules, all C region sequence data obtained for 3547 H chain (Table II) are consistent with the structure of a-positive chains of the d12 allotype (Hill et al., 1967; Fruchter et al., 1970; Pratt & Mole, 1975; Prahl et al., 1969; B. A. Fraser, D. G. Klapper, and A. L. Thunberg, unpublished). This association of identical C regions with both a-positive and a-negative V regions supports the translocation hypothesis of immunoglobulin synthesis (Dreyer & Bennett, 1965).

The existence of additional methionine residues in the V region of 3547 H chain (Figure 7) complicated purification of the products of CNBr cleavage and limited the amount of V region structural data obtained. Inspection of available H chain amino acid sequence data in light of V8 protease specificity [as reported by Houmard & Drapeau (1972) and Johnstone & Mole (1977)] revealed that at least nine susceptible bonds were present in the C-terminal half of the H chain whereas very few susceptible bonds were present in the amino terminal portion.

As anticipated, V8 protease digestion of 3547 H chain yielded a large fragment (V8-1) containing the V region (Figure 2, Table VI). Its N-terminal and C-terminal sequence, obtained from peptides V8-1-CNC-2 and V8-1-TD1-B (Figure 5), and peptide V8-1-CND (Table VI), respectively, showed that it spanned residues 7–258. The observation that two different tryptic peptides derived from fragment V8-1 each contained cysteine residue 204 (fractions 40 and 47, Figure 4) indicates the presence of some lower molecular weight material in the V8-1 preparation. This was presumably a result of partial V8 protease cleavage at glutamic residue 233. Fragment V8-1 is therefore analogous to the CNBr fragment C-1 (Fruchter et al., 1970) but lacks the amino-terminal 6 residues and hence has a free N terminus. V8-protease also removes an N-terminal peptide from a-positive chains (Johnstone & Mole, 1977) and direct automated sequence analysis of fragment V8-1, after additional purification, should be the method of choice for future sequence studies on both a-positive and a-negative H chains, particularly when material is limited in amount. (A total of 2.5 μmol of the 3547 H chain was digested with V8 protease in the present study.)

The specificity of V8 protease (Houmard & Drapeau, 1972; Johnstone & Mole, 1977) was further delineated in the present study. No cleavage of the Glu-Pro bond at positions 157–158 was observed. The structure Pro-Pro-Pro-Glu-Leu (residues 230–234) was only cleaved in 35% of chains (Figure 4), whereas the sequence Thr-Pro-Glu-Val (residues 256–259) was completely cleaved by the V8-protease.

No heterogeneity of 3547 H chain was detected in these studies. The observation of a large CNBr peptide (fraction 42, Figure 3) which contained both cysteine-22 and -96 may be attributed to incomplete cleavage at methionine 64 because of partial alkylation of the methionine residue. However, the existence of an alternative residue at this position cannot be rigorously excluded. The arginine residue at position 78 was only partially cleaved by trypsin (peptides V-8-TF, V8-1-TE, and V8-1-TD1-A; Table VI and Figure 6). No evidence for variation in the sequence around this position was obtained and the reason for this reproducibly incomplete cleavage is unknown.

In the N-terminal 30 residues, which contain most of the subgroup-associated residues in human H chains (Capra & Kehoe, 1975), 3547 H chain is 76.7% homologous to the human V_HIII subgroup (Figure 7). For subgroups V_HI and

V_HII the homology values are 56.7% and 50%, respectively. The homology throughout the V region (excluding hypervariable regions: residues 31–35 and 99–111) between 3547 and V_HIII is 79.3%. Alignment of rabbit a-positive chains with human V_HIII requires a deletion of one residue at the amino terminus and two residues at positions 77 and 78, whereas 3547 is the same length as human chains throughout the V region (Figure 7). This homology confirms and extends the earlier observations of Johnstone & Mole (1977) that were based on the amino terminal 20 residues of pooled a-negative molecules. As earlier noted (Jaton, 1975; Mole, 1975), a-positive H chains are most similar to the human V_HII subgroup with an average homology of 57%, and are least similar to V_HI subgroup with an average homology of 29% (Fraser et al., 1977).

Comparison of 3547 H chain with H chains of allotypes a1, a2, and a3, for the amino-terminal 27 residues, shows greatest similarity exists between the a-negative and a3 (Figure 7). As few as ten mutational events separate genes encoding these sequences, whereas generation of the genes encoding allotypes a1 and a2 from those encoding a-negative chains requires 13 and 16 events, respectively. These are minimum values and allow only one base change for a deletion. In the N-terminal 27 residues, a1 and a2 differ by 10 bases, a2 and a3 by 9 bases, and a1 and a3 differ by 5 bases. Therefore, the difference between a-negative and a3 is of the same order as that between allotypes a1 and a2 or a2 and a3. These limited data agree with the suggestion of Capra et al. (1973) that the V_HIII genes, exemplified in the rabbit by the a-negative population, represent primordial V region genes. It is tempting to speculate that a3 is derived from a-negative and that the a1 and a2 genes are predated by the a3.

In 11 of 14 positions thought to be correlates of group a allotype a difference is noted in one chain whereas the other two are the same. In all but two of the 11 positions the a-negative is the same as two of the a-positive chains. Furthermore, in these 9 positions (5, 8, 12, 15, 66, 68, 71, 88, 89) a3 is identical with a-negative, In fact, the 3547 H chain differs from all three group a allotypes at only three of the 14 positions which are thought to be allotype-related (positions 13, 16, and 72). This emphasizes the importance of these residues to the antigenicity of the molecules but does not preclude involvement of other residues in the allotypic determinants. Combinations of several residues brought into close proximity by folding of the H chains are probably responsible for group a allotype specificities (Mole et al., 1971; Margolies et al., 1977).

The 3547 immunoglobulin was tested for reactivity with antisera directed against the allotypes x32 and y33. Neither antisera reacted with 3547 H chain (K. Knight, personal communication). Therefore, the 3547 chain presumably expresses an x or y allotype different from either of those thus far described (Kim & Dray, 1972). Variation at positions 5, 15, and 19 between the sequences of the 3547 H chain and the a-negative pool may be allotype related. However, further sequence studies on H chains which have defined group x and group y specificities will be required to elucidate the structural correlates of these serological determinants.

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