Amino Acid Sequence of the *N*-Terminal Sixty-Nine Residues of Heavy Chain Derived from a Homogeneous Rabbit Antibody

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The sequence of the *N*-terminal 69 residues of heavy chain from a homogeneous rabbit antibody to type III pneumococcal polysaccharide was determined. The sequence is similar to that found in heavy chains of normal pooled rabbit immunoglobulins of the same allotype Aa1. Two regions of the homogeneous heavy chain (residues 35–46 and 62–69) are very similar to corresponding regions of heavy chains from rabbit Aa2 immunoglobulin, as well as from mouse, guinea-pig and human immunoglobulins. In contrast, residues 47–62 appear to be variable. Comparison in this section with another homogeneous anti-pneumococcal antibody (Strosberg *et al.*, 1972) of related specificity and of the same allotype indicates sequence variation in at least three positions. An antibody to group C streptococcal carbohydrate of allotype Aa2 (Fleischman, 1971) differs by five amino acids in the same region of the heavy chain. Sequence variability between these three antibodies does not occur in homologous positions within this variable section. Allotype-related sequences could not be identified in section 34–65.

Detailed knowledge of the structural basis of antibody specificity requires the determination of the primary structure of both heavy and light chains of the antibody molecule. Sequence determinations of a number of myeloma proteins have shown that L chains* have a constant C-terminal half and a variable *N*-terminal half (Hiltschmann & Craig, 1965). The C-terminal region of the H chain, comprising about 330 residues, is invariant within each sub-class for a given allele, whereas the first *N*-terminal 115 residues are variable (Edelman *et al.*, 1969; Press & Hogg, 1969). Structural studies on myeloma immunoglobulin chains have given insight into the nature of sequence variability, the determination of which was greatly facilitated by their high degree of homogeneity. Conversely, the elucidation of the sequence of rabbit antibody variable regions was hampered by the great structural heterogeneity of most antibody populations (Haber, 1968). However, it was recently shown that rabbit antibodies to streptococcal and pneumococcal vaccines are not infrequently found to be far more homogeneous than antibodies raised to conventional protein antigens (Krause, 1970; Haber, 1971). As these antibodies are usually produced in large amounts, they are ideal for structural studies of the variable regions which play an important role

in antigen binding (Fruchter *et al.*, 1970; Porter, 1971). Homogeneity of streptococcal and pneumococcal antibodies was first demonstrated for the L-chain *N*-termini (Hood *et al.*, 1970; Jaton *et al.*, 1970). The sequence of the *N*-terminal 65 residues of H chain from a partially homogeneous antibody to group C streptococcal carbohydrate was reported recently (Fleischman, 1971).

We have isolated a homogeneous rabbit antibody to type III pneumococci. This antibody of allotype Aa1, designated BS-5, is specific for cellobiuronic acid, the repeating disaccharide unit of the capsular pneumococcal polysaccharide type III. In the present paper we report the sequence of the *N*-terminal 69 residues of H chain derived from antibody BS-5. The sequence of this H chain (referred to throughout this paper as H chain BS-5) is compared with known sequences of H chains derived from two homogeneous rabbit antibodies, as well as to corresponding regions of mouse, guinea-pig and human H chain.

Materials

Trypsin treated with L-1-chloro-4-phenyl-3-tosylamidobutan-2-one and α -chymotrypsin (three times crystallized) were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pronase-P was from Serva, Heidelberg, Germany. Dithiothreitol (A grade) was purchased from Calbiochem Co., Los Angeles, Calif., U.S.A. Iodo[2-¹⁴C]acetic acid (specific radioactivity 32 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Hydriodic

*Abbreviations: IgG, immunoglobulin G; H and L chains, heavy and light chains of IgG; Cl fragment, *N*-terminal half of the rabbit heavy chain; CmCys, Hse, Glu (in amino acid sequences and Tables), *S*-carboxymethylcysteine, homoserine and pyroglutamic acid respectively.

acid (65%, v/v) stabilized with 0.03% (w/v) H_3PO_4 was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Sequencer-grade reagents and solvents used in the automated Edman degradation were obtained from Beckman International, Geneva, Switzerland.

Methods

Antibody formation and preparative methods

Immunization. Rabbit BS-5 was immunized with formalin-treated type III pneumococcal vaccine according to a scheme described in detail by Kimball *et al.* (1971). At the peak of its response, the rabbit was bled twice a week.

Purification of the antibody. This was carried out by means of a specific immunoabsorbent consisting of type III pneumococcal polysaccharide-azo-(bovine serum albumin) conjugate linked covalently to bromoacetyl-cellulose by the method of Jaton *et al.* (1970). The antibody was eluted with 0.2M-acetic acid and was recovered in a yield of 75%. The micro-zone electrophoretic pattern of the isolated antibody disclosed a single band in the γ -globulin region.

The a- and b-locus allotype determinations were kindly performed by Dr. A. S. Kelus. The antibody BS-5 carried a1, b4 allotypes.

Preparation of heavy and light chains. The antibody was reduced with 0.14M-2-mercaptoethanol in 0.5M-tris-HCl buffer, pH 8.2, for 90 min at room temperature and alkylated with 0.2M-monoiodoacetamide at pH 8.2; excess of reagent was removed by dialysis against 0.15M-NaCl and the chains were separated on a Sephadex G-100 column in 1M-acetic acid (Fleischman *et al.*, 1963). The L-chain fraction accounted for 28% of all the material eluted from the column absorbing at 280 nm.

Cyanogen bromide cleavage of the heavy chain. Cyanogen bromide (2.5 g) was added to 440 mg of heavy chain dissolved in 25 ml of 70% (v/v) formic acid. The solution was kept at 4°C for 24 h, diluted 10 times with cold water and freeze-dried. The digest was then redissolved in 1M-acetic acid and run through a Sephadex G-100 column (2 cm $\times$ 200 cm) in the same solvent (Cebra *et al.*, 1968).

Complete reduction of fragment C1. A portion (90 mg) of fragment C1 was fully reduced with dithiothreitol in the presence of 7M-guanidine hydrochloride and alkylated with iodo[2- ^{14}C]acetic acid (O'Donnell *et al.*, 1970).

Isolation of the N-terminal fragment from fragment C1. The reduced and [^{14}C]alkylated fragment C1 preparation was subsequently run on a column of Sephadex G-75 (2 cm $\times$ 200 cm), equilibrated in 0.05M- NH_4HCO_3 and developed with the same solvent, similarly to the method of Wilkinson (1969). The N-terminal fragment was freeze-dried and further

desalted on a Sephadex G-25 column (2 cm $\times$ 120 cm) equilibrated in aq. 0.02M- NH_3 soln.

Analytical methods

Preparative high-voltage paper electrophoresis was carried out at pH 3.5 or 6.5 on Whatman paper no. 1, as described by Press *et al.* (1966). Ninhydrin-negative peptides were detected by the hypochlorite-starch-iodide method (Pan & Dutcher, 1956) and those containing tyrosine, tryptophan or arginine by the 1-nitroso-2-naphthol stain, the Ehrlich reagent or the Sakaguchi stain respectively (Smith, 1960). Peptides were eluted from paper with water and the solutions freeze-dried. Peptides containing [^{14}C]carboxymethylcysteine were detected by radioautography for 20 h by using Kodak Royal Blue Medical X-ray film. Enzymic digestions of 100–300 nmol of peptides were performed for 3–4 h at 37°C, by using 20–60 μg of enzyme in the presence of 0.2–1.0 ml of 1% (w/v) NH_4HCO_3 .

Amino acid analyses were carried out with a Beckman model 121 Amino Acid Analyzer by using the single-column procedure and an accelerated elution program. Hydrolysates of peptides containing homoserine were dissolved in 0.5 ml of pyridine-acetate buffer, pH 6.5, and heated at 110°C for 1 h, before being run on the analyser (Ambler, 1965). Homoserine was eluted between serine and glutamic acid with the first buffer at pH 3.2. Satisfactory analysis could be performed with 20 nmol of amino acids. The yields of serine, threonine, S-carboxymethylcysteine and tyrosine were corrected for losses during HCl hydrolysis by the following factors: Ser 1.15, Thr 1.05, CmCys 1.1 and Tyr 1.08. These factors were computed from analyses of acid hydrolysates of three Bence-Jones proteins of known amino acid sequence.

The sequences of small peptides were determined by the 'dansyl-Edman' procedure described by Gray (1967), and the Dns-amino acids were identified by t.l.c. on polyamide sheets (Selecta; Schleicher and Schüll, Einbeck, Germany), in solvent systems described by Woods & Wang (1967).

The determination of the amino acid sequence of a section of the large C1 fragment of the H chain was performed in the Beckman Sequencer model 890, with 200–300 nmol of fragment C1 dissolved in 0.4 ml of 1M-acetic acid. The program used includes the successive addition of phenylisothiocyanate and Quadrol [NNN'-tetrakis-(2-hydroxypropyl)ethylenediamine-trifluoroacetic acid buffer in aq. propan-1-ol; Wyandotte Chemicals Corp., Wyandotte, Mich., U.S.A.], washing with benzene and ethyl acetate, cleavage of the thiazolinone derivative with heptafluorobutyric acid, and its extraction with chlorobutane (Edman & Begg, 1967). Dithiothreitol (0.5 mm) was added to the chlorobutane, as the

presence of the reducing agent significantly increased the yield of phenylthiohydantoin-amino acids, particularly that of serine (Hermodson *et al.*, 1970). The resulting thiazolinone derivatives of the amino acid residues were converted into the corresponding phenylthiohydantoin derivatives by treatment with 0.2ml of 1M-HCl at 80°C for 10min in a nitrogen atmosphere. The phenylthiohydantoin-amino acid derivatives were extracted into ethyl acetate and were then assayed by (1) g.l.c. (Pisano & Bronzert, 1969) on a Beckman GC-45 gas chromatograph, and (2) amino acid analysis after conversion of the phenylthiohydantoin derivatives into free amino acids by hydrolysis with 65% HI for 20h at 125°C (Smithies *et al.*, 1971). In this procedure, serine, threonine and isoleucine were identified by amino acid analysis as alanine, α -aminobutyric acid and alloisoleucine+isoleucine respectively. Phenylthiohydantoin-arginine was not recovered in satisfactory yield in our hands; it was detected by the phenanthrenequinone spot test (Yamada & Itano, 1966), as was phenylthiohydantoin-histidine by the Pauli test (Smith, 1960). Phenylthiohydantoin-tryptophan, completely destroyed by acid hydrolysis, was identified by g.l.c. The distinction between free and amidated side-chain carboxyl groups was done by g.l.c. analysis of the

silylated phenylthiohydantoin-dicarboxylic acids (Pisano & Bronzert, 1969).

Radioactivity was measured in vials containing 10ml of scintillation liquid (Bray, 1960) in a Nuclear-Chicago liquid-scintillation counter.

Results

Isolation of the N-terminal cyanogen bromide fragment from heavy chain BS-5

The N-terminal half of H chain BS-5, C1, resulting from splitting H chain with cyanogen bromide (Cebra *et al.*, 1968), was obtained as described in the Methods section. The elution diagram is illustrated in Fig. 1. It was found that the amino acid composition of the top fractions under the first two peaks in Fig. 1 was identical; the material under the hatched area in Fig. 1 was pooled and accounted for 45% of the absorbance at 280nm of the total cyanogen bromide digest. It was referred to as fragment C1. There are 1.7 residues of homoserine per molecule of fragment C1, based on 17 leucine residues/molecule

Table 1. *Amino acid composition of the cyanogen bromide fragments isolated from rabbit H chain BS-5 (allotype Aa1)*

Values are residues per molecule of peptide. Values for fragment C1 are based on 17 residues of leucine (Cebra *et al.*, 1968).

Amino acid	Fragment C1	N-Terminal fragment
Lys	10.6	0.0
His	1.7	0.0
Arg	6.1	1.1
Asp	13.3	1.0
Thr	29.0	5.4
Ser	28.2	5.6
Glu	13.4	3.1
Pro	20.2	2.2
Gly	18.9	4.0
Ala	12.4	0.0
$\frac{1}{2}$ Cys*	3.8	0.0
Val	21.3	2.9
Ile	5.7	0.0
Leu	17.0	3.9
Tyr	7.3	0.9
Phe	6.8	1.0
CmCys	2.9	0.8
Hse	1.7	0.8
Trp	+†	-†
Total residues‡	220.3	32.7

* Value not corrected for losses during hydrolysis.

† Measured with Ehrlich reagent.

‡ Excluding tryptophan.

Fig. 1. *Elution profile of a cyanogen bromide digest of H chain BS-5*

The Sephadex G-100 column (2cm×200cm) was developed in 1M-acetic acid. For other details see the text. —, E_{280} . Fragments C1 and C2 contain the N-terminal half of the H chain; fragments C3–C5 are peptides derived from the constant C-terminal half of the chain.

(Table 1). This indicates the presence of one homoserine residue within fragment C1, in addition to the C-terminal homoserine at position 253 (Fruchter *et al.*, 1970). Fragment C1 was thus split into two fragments, which were held together by a disulphide bond. The smaller N-terminal fragment was separated from the larger C-terminal peptide after full reduction, alkylation with iodo[^{14}C]acetate and chromatography on a column of Sephadex G-75. Three fractions, A, B and C, were obtained (Fig. 2): they contained the large cyanogen bromide fragment (A), the N-terminal fragment (B) and excess of salts (C) respectively. The N-terminal fragment was desalted on a column of Sephadex G-25 in aq. 0.02M- NH_3 soln. and freeze-dried. It was recovered in 63% yield. From its amino acid composition, shown in Table 1, this 33-residue peptide was considered pure enough for sequence analysis.

Sequence of the N-terminal fragment of H chain BS-5

The dansylation technique did not reveal any amino acids, suggesting that the N-terminal residue was blocked. The N-terminal peptide was fragmented by digestion with α -chymotrypsin and the mixture fractionated by paper electrophoresis at pH6.5. Four peptides were detected (Table 2). Peptide Ch-1 contained five amino acids; the 'dansyl-Edman' procedure established the sequence Ser-Leu-Ser-Ser-

Tyr. All radioactivity of the original N-terminal fragment was in peptide Ch-2. Eight cycles of 'dansyl-Edman' degradation were carried out, indicating the

Fig. 2. Isolation of the N-terminal fragment from fragment C1

The Sephadex G-75 column (2cm $\times$ 200cm) was developed in 0.05M- NH_4CO_3 . —, E_{280} ; ·····, E_{230} ; - - - - -, radioactivity (c.p.m./50 μl of eluate). See the text for details. The horizontal bar indicates the position of the N-terminal fragment.

Table 2. Amino acid composition and properties of constituent peptides of the N-terminal fragment of H chain BS-5 (allotype Aa1)

Values are residues per molecule of peptide. Numbers in parentheses are integral values confirmed by sequence analysis. Tryptic peptides were derived from peptide Ch-3; peptides P1 and P2 were obtained from a Pronase digest of peptide T1. Mobilities are expressed relative to Asp (= -1.0). Yields are based on 275nmol of starting material (N-terminal cyanogen bromide fragment).

Amino acid	Chymotryptic peptides				Tryptic peptides		Pronase peptides	
	Ch-1	Ch-2	Ch-3	Ch-4	T1	T2	P1	P2
Arg	—	—	0.9 (1)	—	1.0 (1)	—	—	0.8 (1)
Asp	0.1	—	—	1.0 (1)	—	—	—	—
Thr	—	2.8 (3)	2.2 (2)	—	—	2.1 (2)	—	—
Ser	2.8 (3)	1.1 (1)	1.6 (2)	—	2.2 (2)	—	1.3 (1)	1.2 (1)
Glu	—	—	3.0 (3)	—	3.2 (3)	—	2.0 (2)	1.0 (1)
Pro	—	—	1.7 (2)	—	—	1.9 (2)	—	—
Gly	0.1	1.1 (1)	2.6 (3)	—	2.1 (2)	1.1 (1)	—	1.8 (2)
Val	—	0.8 (1)	2.1 (2)	—	0.8 (1)	1.0 (1)	0.9 (1)	—
Leu	1.0 (1)	1.0 (1)	2.0 (2)	—	—	2.0 (2)	—	—
Tyr	1.0 (1)	—	—	—	—	—	—	—
Phe	—	0.8 (1)	—	—	—	—	—	—
CmCys	—	0.8 (1)	—	—	—	—	—	—
Hse	—	—	—	0.9 (1)	—	—	—	—
Total residues	5	9	17	2	9	8	4	5
Mobility at pH6.5	0	-0.30	-0.38	-0.68	—	—	-0.79	0
Yield (%)	40	67	63	27	38	47	18	31

following sequence: Thr-Leu-Thr-CmCys-Thr-Val-Ser-Gly-Phe. The position of *S*-carboxymethylcysteine in the nonapeptide was confirmed by the removal of 78% of the radioactivity in the peptide after the fourth step. Peptide Ch-4 was a dipeptide containing aspartic acid and homoserine. From its mobility at pH 6.5 (Table 2) it was concluded that one of the residues is aspartic acid. Since homoserine is expected to be the *C*-terminal residue of the 33-residue peptide, the sequence of peptide Ch-4 should be Asp-Hse.

The heptadecapeptide Ch-3 was ninhydrin-negative and stained for arginine. It was further fragmented with trypsin and the digest was separated by paper electrophoresis at pH 3.5. Two peptides, T1 and T2, were obtained. Peptide T1 moved slightly slower than neutral amino acid at pH 3.5, and had a blocked *N*-terminal residue. It was digested with Pronase and the mixture fractionated by paper electrophoresis at pH 6.5. Two peptides, P1 and P2, were obtained (Table 2). Peptide P1 was a ninhydrin-negative tetrapeptide, which stained with starch-iodide reagent; its mobility at pH 6.5 (-0.79) and its calculated molecular weight indicated that it carried two negative charges at pH 6.5. This suggests that one of the two glutamic acid residues found by amino acid analysis is glutamic acid (Offord, 1966); the other is assumed to be pyroglutamic acid. No sequence determination was attempted because of the small amount of peptide available. However, two blocked *N*-terminal peptides were isolated from a Pronase digest of 15mg of fragment C1. They had the same mobility at pH 3.5 and composition as those described by Wilkinson *et al.* (1966), i.e. (L⁺Glu,Ser) and (L⁺Glu,Ser,Val,Glu). It was therefore concluded that the partial sequence of peptide P1 is L⁺Glu-Ser(Val,Glu). The arginine-containing peptide P2 had the sequence Glu-Ser-Gly-Gly-Arg. The second tryptic peptide, T2, was an octapeptide containing two proline residues. Seven cycles of Edman degradation indicated the sequence Leu-Val-Thr-Pro-Thr-Pro-Gly-Leu.

The sequence of the 33-residue peptide is summarized in Fig. 3. The alignment of the various constituent peptides is based on their homology with the sequence of the *N*-terminal fragment of pooled Aa1 H chains (Wilkinson, 1969). It follows from this alignment that this peptide is the *N*-terminal peptide of H chain BS-5.

Automated sequential degradation of a variable region of fragment C1

The *N*-terminal residue of H chain BS-5 was found to be unreactive in the Edman degradation. Cyanogen bromide cleaved C1 between residues 33 (methionine) and 34. It was thus expected that the sequence determination of fragment C1 would proceed starting with residue 34. This proved to be the case. The sequence of fragment C1 from residues 34 to 69 was established by means of the protein sequencer, by using 5–8mg of fragment C1 prepared as described in the Methods section. The overall repetitive yield in three sequencing experiments varied from 92 to 93%, and the absolute yield of phenylthiohydantoin-glycine at the first step ranged between 33 and 42%, which is of the same order of

Fig. 3. Amino acid sequence of the *N*-terminal fragment of H chain BS-5

For experimental details see the text. Ch, T and P are chymotryptic, tryptic, and Pronase peptides respectively. →, Amino acid residues identified by the 'dansyl-Edman' procedure; ⇨, identified as dansyl residue without hydrolysis.

Fig. 4. Sequence of the first 69 amino acid residues of the H chain BS-5

The sequence comprises the *N*-terminal cyanogen bromide fragment (residues 1–33) and part of the C1 fragment subjected to automated Edman degradation (residues 34–69).

magnitude as that obtained for Bence-Jones proteins. A single amino acid comprised more than 90% of the residues found in each cycle of degradation. The amino acid sequence of the *N*-terminal 69 residues of H chain BS-5 is presented in Fig. 4.

Discussion

The *N*-terminal sequence of the first 69 residues of H chain BS-5 of allotype Aa1 is described. The evidence for homogeneity of the antibody in the region so far studied is based on (1) the unique sequence in the section 34–69 of the fragment C1 determined by the automated sequencer, (2) the relatively high yield of the *N*-terminal 33-residue peptide derived from the CNBr-treated H chain (63%), and (3) the single sequence in the *N*-terminal 33 positions of the corresponding L chain (J. C. Jaton, unpublished work). It is therefore likely that the *N*-terminal sequence 1–69 reported here is representative of all molecules present in this preparation.

Fig. 5 shows a high degree of similarity between sequences of H chain BS-5 and H chain of pooled IgG of the same allotype (Wilkinson, 1969; Mole *et al.*, 1971). However, the alternative residues that are found at positions 3, 23, 32, 34, 46, 49–51 and 60 (BS-5 numbering) of the pooled material are not found in the BS-5 material. The extensive homology of distinct segments of H chain BS-5 with corresponding segments derived from the H chains of different species is striking: section 35–46 is invariant for rabbit H chains of allotype Aa1 and Aa2, except for position 42, where aspartic acid is present in H chain of antibody R 26–90. Similarly, human myeloma H chain Nie (Sub-group VH III), mouse myeloma MOPC 173 H chain and guinea-pig γ 2 H chain are identical in that section with H chain BS-5, except for position 36, where isoleucine is found in guinea-pig H chain. Mouse myeloma MOPC 315 H chain, however, differs in four positions from the sequence of H chain BS-5 (Fig. 5). Residues 62–69 appear to be rather invariant in rabbit H chains, whereas differences are found between different H chains of man and mouse. In section 62–69, guinea-pig H chain displays only one amino acid interchange.

Recent reports indicate that allotype-related residues are included in the *N*-terminal 33-residue peptide of the rabbit H chain, but are not demonstrable in section 34–65 (Wilkinson, 1969; Mole *et al.*, 1971). Although results on H chains of individual antibodies are thus far insufficient to allow definite conclusions, our results do not contradict this assumption; allotype-related sequences are unlikely to occur in section 49–61, which shows variability between different Aa1 chains (discussed below), or in the more constant sections 35–48 and

62–65. The Lys-Asp and Gly-Ser interchanges in positions 42 and 64 respectively are unlikely to be allotype-related (Mole *et al.*, 1971).

In contrast, section 47–62 displays many alternative residues. The variability in the rabbit antibody chains seems to be restricted to residues 49–61. In that section, H chain BS-5 differs from H chain of antibody 325 to type VIII pneumococci, shown here for comparison, in at least three positions (Strosberg *et al.*, 1972). The specificity of the two antibodies is similar as antibody 325 to type VIII pneumococci was found to cross-react entirely with type III pneumococcal polysaccharide (J. C. Jaton & E. Haber, unpublished work). On the other hand, antibody R26–90, specific for an unrelated antigenic determinant, i.e. group C streptococcal carbohydrate (Fleischman, 1971), shows five amino acid interchanges (Fig. 5). These are not the positions in which the two anti-pneumococcal chains differ. The few sequence results so far available in that section do not yet allow determination of the correlation between sequence variability and binding specificity. However, it is noteworthy that affinity labelling of mouse myeloma protein MOPC 315 'tagged' lysine-54 (51 in BS-5 numbering) in the H chain; the labelled tryptic peptide was found to occupy residues 44–63 (Fig. 5), suggesting that part of the combining site is formed by amino acid residues in this variable region (Haimovich *et al.*, 1972). Similarly, the results of Birshtein & Cebra (1971) suggest that this section is quite heterogeneous in normal guinea-pig γ 2 chain, whereas purified anti-dinitrophenyl antibodies prepared in the same species display a marked restriction in the number of alternative amino acids in many positions of that section (indicated by V in Fig. 5). Taken together, the results discussed here make section 47–62 a possible region of H chain which may be involved in antigen binding.

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