The V-Region Sequence of the H Chain from a Third Rabbit Anti-Pneumococcal Antibody

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The amino acid sequence of the V (variable) region of the heavy (H) chain of rabbit antibody BS-1, raised against type III pneumococcal vaccine, is reported. Together with the sequence data of the V region of the light (L) chain previously determined [Jaton (1974a) *Biochem. J.* **141**, 1–13], the present work completes the analysis of the V domain of the homogeneous antibody BS-1. The V domains ($V_L + V_H$ regions) of this antibody are compared with those of two other anti-(type III) pneumococcal antibodies BS-5 and K-25 [Jaton (1975) *Biochem. J.* **147**, 235–247]. Except for the second hypervariable section of the L chains, these antibodies have very different sequences in the hypervariable segments of the V domains. Within the third hypervariable region of the H chain, each antibody has a different length: BS-1 is three amino acids shorter than K-25 and two amino acids shorter than BS-5. When the sequences in that section are aligned for maximal homology, only two residues, glycine-97 and leucine-101, are common to the three antibodies. On the basis of the amino acid sequences of these three anti-pneumococcal antibodies, the results do not support the concept of a simple correlation between primary structure in the hypervariable sections (known to determine the shape of the combining site) and antigen-binding specificity.

The amino acid sequence of the variable (V) domain of the immunoglobulin molecule [*N*-terminal 110–120 residues of light (L) and heavy (H) chains] consists of hypervariable and non-hypervariable, or 'framework' regions (Kehoe & Capra, 1971; Wu & Kabat, 1970). V_L and V_H regions have three to four hypervariable sections, each containing 5–15 amino acid residues (Capra & Kehoe, 1975); these segments are separated within sequences of the polypeptide chain, but are spatially brought near to each other in the final quaternary structure to form the antigen-binding site of the antibody molecule (Poljak, 1975). Affinity-labelling studies of a few myeloma proteins with hapten-binding activity (Givol, 1973) as well as the inspection of three-dimensional molecular models constructed on the basis of X-ray-diffraction data strongly suggest the involvement of distinct amino acids from hypervariable sections of both chains in antigen binding (Davies *et al.*, 1975).

The establishment of a correlation between primary structure and antigen-binding specificity is a major goal in the understanding of structure-function relationships; further, the determination of antibody V-region sequences has a direct impact on the possible mechanisms by which diversity of immunoglobulins is generated. One of the decisive issues is whether V regions of antibodies are coded for by structural genes in the germ line. Evidence for

the inheritance of κ L chain subgroup (Braun & Jaton, 1973) or of idiotypic determinants (Kindt *et al.*, 1973) in anti-polysaccharide antibodies raised in selectively bred rabbits has been reported. Further support for this observation comes from the demonstration of fairly uniform amino acid sequences in the first and second hypervariable regions of anti-hapten pooled H chains from inbred guinea pigs (Cebra *et al.*, 1974). Similarly, individuals within certain inbred strains of mice produced antibodies to phosphorylcholine or to arsonate haptens which exhibited the same or very similar binding-site structures (Claffin & Davie, 1974; Capra *et al.*, 1975a).

Complete structural data on the V domain of elicited rabbit antibodies are still limited, especially with respect to the H chains of these antibodies; assessment of the extent of sequence uniformity in hypervariable segments of the V_H region will be of considerable interest, since the H chain seems to play, at least in some systems, a more important role than the L chains in determining the specificity of the antibody (Porter, 1971; Barstad *et al.*, 1974a). This is consistent with the suggestion that antibodies of a defined specificity with shared idiotypic determinants may contain the same type of H chain in combination with different types of L chains (Capra *et al.*, 1975b).

The present paper describes the amino acid sequence of the V region of the H chain derived from

the homogeneous rabbit antibody BS-1, directed to type-III pneumococcal polysaccharide. The determination of the sequence of this H chain of allotype a_2 , and that of the corresponding V_L region previously reported (Jaton, 1974a), completes the analysis of the primary structure of the Fv domain of antibody BS-1.

Experimental

Materials

Radiochemicals and reagents used for the isolation of peptides and for the manual and automated sequence determination were described previously (Jaton, 1974a).

Antibody purification

Rabbit antibody BS-1 was isolated with an immunoabsorbent followed by preparative agarose-block electrophoresis (Jaton, 1974a).

Cleavage of H chain BS-1 with CNBr

This was carried out by adding solid CNBr (twice the weight of the H chain) to the H chain dissolved in 70% (v/v) formic acid. The *N*-terminal half of H chain BS-1 (CNBr fragment C1) was isolated by Sephadex G-100 chromatography in the presence of 1M-acetic acid (Jaton *et al.*, 1973). The complete reduction and alkylation of CNBr fragment C1 with iodo[2- 14 C]acetic acid was performed as described by Jaton & Braun (1972). The citraconylation of large CNBr fragments from fragment C1 and the purification of the arginine-containing peptides have been carried out as reported (Jaton, 1974a,b).

Analytical methods

Amino acid composition, enzymic digestions, preparative high-voltage paper electrophoresis and sequence analysis of large peptides in the Beckman sequencer have been described in full elsewhere (Jaton, 1974a,b,c,d). The sequence determination of tryptic peptides and of non-polar peptides was facilitated by the use of 4-sulphophenyl isothiocyanate (Braunitzer *et al.*, 1971) and 2-amino-1,5-naphthalenedisulphonic acid respectively to minimize losses of peptides (Foster *et al.*, 1973). In some cases, t.l.c. of subnanomole amounts of phenylthiohydantoin derivatives of amino acids on polyamide sheets was used as described (Summers *et al.*, 1973).

Results

Primary structure of the V region of H chain BS-1: characterization of CNBr fragments

The *N*-terminal half of H chain BS-1 (CNBr fragment C1) was obtained, after cleavage of the H chain with CNBr, in 82% yield. This fragment was

subjected to automated Edman degradation; two major residues in the same yield were obtained at each of the first six positions, indicating the presence of two mixed sequences; by homology with the known sequence of H chain K-25, which also carries allotype a_2 as does chain BS-1 (Jaton, 1975), the two sequences are probably Asx-Ser-Leu-Thr-Ala-Glx and Asp-Val-Trp-Gly-Pro-Gly, thus suggesting the presence of two internal methionine residues, one at position 78, the other at the beginning of C_{H1} domain. The resulting CNBr peptides were separated from each other by Sephadex G-100 chromatography after full reduction and alkylation of the intrachain disulphide bonds (Fig. 1); four fractions were obtained. CNBr fraction I was shown to have the same *N*-terminal sequence as the C_{H1} domain: Asp-Val-Trp-Gly-Pro-Gly (Pratt & Mole, 1975; Jaton, 1975), which indicates that fraction I contains the C_{H1} domain of the antibody BS-1 (see below). Fraction II, recovered in 65% yield, was shown to contain about 76 amino acids, 0.8 *S*-carboxymethylcysteine residue and 0.6 homoserine residue/molecule (Table 1). The *N*-terminal residue appeared to be inaccessible to Edman reagent; this suggests that fraction II is the *N*-terminal 78-residue peptide of the H chain. The amino acid composition of fraction III indicated the presence of 24 amino acids, and its *N*-terminal sequence determined by dansyl-Edman procedure was Asx-Ser-Leu-Thr, which is characteristic of the a_2 sequence between positions 79 and 82. This fraction, obtained in 58% yield, contains peptide 79-102 and therefore follows fraction II sequentially. Fraction IV was shown to contain excess of salts and iodo[2- 14 C]acetate; no peptides could be detected in significant amount after desalting this fraction on Sephadex G-10. Thus fractions II and III comprise the entire V region of H chain BS-1, whereas fraction I constitutes the C_{H1} domain of fragment C1.

Fig. 1. Separation of large homoserine-containing peptides from a CNBr digest of reduced and [2- 14 C]alkylated fragment C1 of H chain BS-1

The column (2.5cm $\times$ 190cm) of Sephadex G-100 was developed in 5M-guanidine hydrochloride, pH 3.5. —, E_{280} . See the text for details.

Table 1. Amino acid composition of CNBr peptides and of some chymotryptic peptides

Values in parentheses are integral values confirmed by sequence analysis. All chymotryptic peptides are derived from fraction II. Mobilities are expressed relative to arginine (= +1.0) [the mobility of arginine (relative to aspartic acid = -1.0, corrected for endosmosis) is 0.91]. Hse, homoserine; ND, not determined.

Amino acid	Composition (residues/mol of peptide)							
	CNBr peptides		Chymotryptic peptides					
	Fraction II	Fraction III	Ch _{N1} -A ₁	Ch _{N4} -A ₁	Ch ₆ -A ₁	Ch ₂ -A ₂	Ch ₃ -A ₂	Ch _{N1} -A ₂
Lys	3.6 (4)					1.0 (1)	1.0 (1)	
His		0.9 (1)						
Arg	3.2 (3)	1.2 (1)			1.0 (1)			
CmCys	0.8 (1)	0.8 (1)						
Asp	3.6 (3)	2.2 (2)						
Thr	12.8 (11)	4.5 (4)		1.0 (1)			0.9 (1)	
Ser	11.7 (13)	1.2 (1)	3.2 (3)				2.9 (3)	1.2 (1)
Glu	7.0 (6)	0.6 (1)						
Pro	2.3 (2)	—						
Gly	7.3 (7)	1.9 (2)					1.3 (1)	
Ala	4.3 (4)	2.5 (3)		1.0 (1)		1.0 (1)		1.0 (1)
Val	4.2 (5)	0.2 (0)		0.8 (1)	1.0 (1)			
Ile	3.0 (3)	0.9 (1)						
Leu	6.8 (7)	3.0 (3)	1.0 (1)					
Tyr	2.7 (3)	1.8 (2)	0.9 (1)				0.9 (1)	1.0 (1)
Phe	2.4 (2)	1.1 (1)						
Hse	0.6 (1)	0.6 (1)						
Trp*	ND	—		+(1)				+(1)
Total residues	76.3	23.4	5.1	2.8	2	2	7.0	3.2
Mobility at pH 6.5	ND	ND	0	0	+0.78	+0.82	+0.30	0

* Qualitative determination with Ehrlich reagent.

Characterization of the N-terminal CNBr fragment (fraction II)

As the amino acid composition of this peptide suggested the presence of a limited number of arginine residues (Table 1), citraconylation followed by trypsin digestion was attempted to split this fragment into large arginine-containing peptides amenable to automated Edman degradation. A typical separation of a tryptic digest of citraconyl-fraction II is illustrated in Fig. 2. Three major peaks could be seen, designated CT-A₁, CT-A₂ and CT-A₃. The radioactivity profile indicates that CT-A₁ contained the bulk of the radioactivity in the digest. All three fractions were cross-contaminated. Fractions CT-A₁ and CT-A₂ (hatched areas in Fig. 2) were twice rechromatographed separately on Sephadex G-50 in 0.05M-NH₄HCO₃; under these conditions, the percentage contamination of fraction CT-A₂ by CT-A₁ was estimated to be less than 15%, as measured by the total amount of radioactivity present in fraction CT-A₂.

Fraction CT-A₁ was shown by the dansyl-Edman procedure to be blocked and the only fraction containing radioactivity in the digest, thus showing that CT-A₁ is the N-terminal portion of fraction II.

The N-terminal sequence of fraction CT-A₂ was

Fig. 2. Isolation of arginine-containing peptides from a tryptic digest of citraconylated, reduced and [2-¹⁴C]-alkylated fragment from fraction II

The column (1.2cm×250cm) of Sephadex G-50 (fine grade) was developed in 0.05M-NH₄HCO₃. The material under the hatched area in the first two peaks (fractions CT-A₁ and CT-A₂) was taken for re-chromatography under the same conditions. Fraction CT-A₃ was further characterized by paper electrophoresis. For details, see the text. —, E₂₃₀; ---, radioactivity (c.p.m./20 μl of eluate).

Table 2. Amino acid composition of selected tryptic peptides from the V region of H chain antibody BS-1

Values in parentheses are integral values confirmed by sequence analysis. The tryptic peptides are derived from citraconyl fragments A₁, A₂ and A₃ and from CNBr fraction III. Mobilities are expressed relative to arginine (= +1.0) [the mobility for arginine (relative to aspartic acid = -1.0, corrected for endosmosis) is 0.91]. Hse, homoserine; ND, not determined.

Amino acid	Peptide ...	Composition (residues/mol of peptide)				
		T _{N2} -A ₁	T _A -A ₂	T _B -A ₂	CT-A _{3a}	T ₂ -FrIII
Lys		1.0 (1)		0.8 (1)		
His						0.9 (1)
Arg			1.0 (1)		1.0 (1)	
Asp						0.2 (0)
Thr				1.1 (1)	2.1 (2)	1.2 (1)
Ser		1.1 (1)	1.0 (1)	4.3 (4)	1.1 (1)	
Glu		1.0 (1)				
Gly				1.3 (1)		2.3 (2)
Ala				2.0 (2)		0.3 (0)
Val		0.9 (1)				
Ile					0.9 (1)	0.7 (1)
Leu						2.0 (2)
Tyr				1.9 (2)		1.1 (1)
Phe						0.3 (0)
Hse						0.6
Trp*				+ (1)		—
Total residues		4	2	11.4	5.1	9.6
Mobility at pH 6.5		0	+0.80	+0.23	+0.44	0
						ND

* Qualitative determination with Ehrlich reagent.

Tryptic peptides			Positions in the sequence
T _{N2} -A ₁	□	Glu-Ser-Val-Lys	1-4
T _A -A ₂		Ser-Arg	64-65
T _B -A ₂		Ser-Ser-Gly-Ser-Thr-Tyr-Tyr-Ala-Ser-Trp-Ala-Lys	52-63
CT-A _{3a}		Ser-Thr-Ile-Thr-Arg	66-70
T ₂ -FrIII		Ile-Thr-His-Gly-Tyr-Leu-Gly-Leu-Met	94-102
Chymotryptic peptides	Ch _{N1} -A ₁	Ser-Leu-Ser-Ser-Tyr	27-31
	Ch _{N4} -A ₁	Thr-Val-Ala-Trp	32-35
	Ch ₆ -A ₁	Val-Arg	36-37
	Ch ₂ -A ₂	Ala-Lys	62-63
	Ch ₃ -A ₂	Lys-Ser-Ser-Gly-Ser-Thr-Tyr	51-57
	Ch _{N1} -A ₂	Tyr-Ala-Ser-Trp	58-61

Fig. 3. Sequences of selected peptides from the V region of H chain BS-1

The amino acid sequence was determined by the dansyl-Edman procedure (→), by digestion with carboxypeptidases A and B (←) and subsequent identification on the amino acid analyser, and by automated analysis with a volatile dimethylbenzylamine buffer and the use of 4-sulphophenyl isothiocyanate for peptide T_B-A₂. —, Dansyl residue identified without hydrolysis. The amino acid compositions and electrophoretic mobilities of the peptides are given in Tables 1 and 2.

□ Glu, 5-pyrrolidone-2-carboxylic acid.

Fig. 4. Amino acid sequence of fraction II, the CNBr fragment 1-78 of H chain BS-1

This fragment comprises the citraconyl-peptides CT-A₁, CT-A₂, CT-A₃ and CT-A_{3INT}. The automated sequence determination of the tryptic digest of decitraconylated fraction CT-A₁ was carried out with 450 nmol of material with the dimethyl/benzylamine programme; that of peptide CT-A₂ was done with 200 nmol of peptide. CT, citraconyl; T, Ch, tryptic and chymotryptic respectively. The subscript N in the designation of peptides indicates that the peptide is neutral at pH 6.5. The presence of another tryptophan residue between positions 61 and 62 cannot be ruled out with certainty under the experimental conditions used here. However, the existence of only one tryptophan at position 61 is likely by homology with other rabbit H-chain sequences known so far. See also the text for details. --□_{GLA}, 5-Pyrrolidone-2-carboxylic acid.

found to be Gln-Ala-Pro-, and digestion by carboxypeptidases A and B indicated the C-terminal sequence to be Ser-Arg. By homology with H chain K-25 (Jaton, 1975), the citraconyl-peptide CT-A₂ extends between position 38 and 65 (as indicated in Fig. 4).

Fraction CT-A₃ was desalted on a column of Sephadex G-10 equilibrated with 0.02M-NH₃ and the material subjected to high-voltage paper electrophoresis at pH3.5 and 6.5. Two small peptides, designated CT-A_{3a} and CT-A_{3NI}, were resolved and eluted. Their amino acid compositions and electrophoretic mobilities are presented in Table 2. As the octapeptide CT-A_{3NI} contains a homoserine residue, it must be the C-terminal peptide of fraction II. Because of an insufficient amount of pure peptide, its sequence could not be established. The sequence of the other peptide CT-A_{3a} is shown in Fig. 3.

Sequence of peptide CT-A₁

Since the arginine-containing fragment CT-A₁ is the N-terminal part of an H chain of allotype a₂, it was expected that it contains two constant lysine residues, one at position 4 from the N-terminus, the other being adjacent to a proline residue, by homology with known sequences of a₂ H chains (Fleischman, 1971; Jaton & Haimovich, 1974). Thus tryptic cleavage would be restricted to lysine-4, and, as the N-terminal fragment is blocked, automated

sequence analysis of the whole digest would yield a single sequence starting with the residue located in position 5 of the H chain. This proved to be the case; as shown in Fig. 4, a tryptic digest of decitraconylated fragment CT-A₁ yielded a single sequence of 24 residues. The N-terminal tetrapeptide T_{N2}-A₁ was analysed by conventional methods (Fig. 3). The sequence of the C-terminal part of fragment CT-A₁ was established by the isolation and characterization of three chymotryptic peptides, Ch_{N1}-A₁, Ch_{N4}-A₁ and Ch₆-A₁. Their amino acid compositions and sequences are reported in Table 1 and Fig. 3 respectively. Since the dipeptide Ch₆-A₁, Val-Arg, overlaps the C-terminal sequence of fragment CT-A₁, Trp-Val-Arg, determined by digestion by carboxypeptidases A and B, peptide Ch₆-A₁ is C-terminal to fragment CT-A₁. The sequence of the pentapeptide Ch_{N1}-A₁ overlaps residues 27 and 28, thus allowing its order and that of peptide Ch_{N4}-A₁ between Ch_{N1}-A₁ and the C-terminal dipeptide Ch₆-A₁ (Fig. 4).

Sequence of peptide CT-A₂

The N-terminal portion of fragment CT-A₂ was analysed in the sequencer by using the volatile dimethylbenzylamine programme (Jaton, 1974d). The slight contamination of this peptide by the N-terminal fragment CT-A₁ did not cause sequence ambiguity, since the contaminating peptide is unreactive to Edman reagent. Only the N-terminal

Fig. 5. Sequences of the CNBr fragments in fractions I and III

(a) The N-terminal 19 positions in fraction III were analysed by subjecting 250nmol of peptide, previously treated with 2-amino-1,5-naphthalenedisulphonic acid to automated degradation. The sequence of the C-terminal part of fraction III was completed after isolation of the tryptic peptide T₂-FrIII. Fraction III comprises the rest of the V_H region of the H chain. (b) The N-terminal sequence of the CNBr peptide of fraction I was determined by using the Quadrol programme as described by Jaton (1974a). By homology with rabbit sequences of the constant region (Pratt & Mole, 1975), fraction I represents the C_{H1} domain of H chain BS-1.

Fig. 6. Amino acid sequence of the V domain of rabbit antibody BS-1

The sequence of the V_L region is from Jaton (1974a). The V domain of antibody BS-1 comprises the V regions of the L chain (residues 1-109) and of the H chain (residues 1-102). Deletions (---) are introduced to maximize the homology. Residues underlined belong to the hypervariable sections of the V domain. --[Glu, 5-Pyrrolidone-2-carboxylic acid.

15 residues could be determined (Fig. 4) because of the small amount of peptide with a free *N*-terminal α -amino group: the absolute yield of the phenylthiohydantoin derivative of glutamine at step 1 was 20%, on the basis of about 200 nmol of fraction CT-A₂. This low yield is likely to be due to partial cyclization of the glutamine residue into pyrrolidone-carboxylic acid, although precautions were taken to avoid acid treatment during the isolation of peptide CT-A₂. Tryptic and chymotryptic digestions of this peptide were carried out to complete the sequence analysis. The characterization of the tryptic peptides T_b-A₂ and T_a-A₂ is given in Table 2 and the corresponding sequences in Fig. 3. The *C*-terminal sequence of fraction CT-A₂, Ala-Lys-Ser-Arg, permits the identification of peptide T_a-A₂ as the *C*-terminal dipeptide of fraction CT-A₂, and peptide T_b-A₂ as the *N*-terminal segment relative to peptide T_a-A₂ (Fig. 4). One tryptophan residue per molecule of peptide T_b-A₂ has been qualitatively evaluated (Tables 1 and 2; Fig. 3). The presence of another tryptophan residue following tryptophan-61 (Fig. 4) cannot be ruled out with certainty. However, the existence of only one tryptophan (at position 61) is very likely by homology with other rabbit H-chain sequences known thus far. Three chymotryptic peptides designated Ch₃-A₂, Ch_{N1}-A₂ and Ch₂-A₂ were also isolated from that region and characterized (Table 1, Fig. 3): peptide Ch₃-A₂ provides the overlap with the *N*-terminal sequence of fraction CT-A₂ determined with the sequencer (Fig. 4).

The amino acid sequence of the CNBr fraction II is summarized in Fig. 4. This 78-residue peptide comprises the sequences of the citraconyl peptides CT-A₁, CT-A₂, CT-A_{3a} and CT-A_{3N1}, in that order.

Characterization of CNBr fraction III

The sequence in the *N*-terminal 19 residue positions of the peptide contained in fraction III is reported in Fig. 5(a). The *C*-terminal sequence was determined after isolating the *C*-terminal tryptic homoserine-containing peptide T₂-FrIII by paper electrophoresis at pH 6.5 and 8.9. Its amino acid analysis is shown in Table 2 and its sequence in Fig. 3. Fig. 5(a) summarizes the structural data and indicates that the CNBr peptide of fraction III extends from position 79 to 102 by homology with the known sequence of rabbit H chain K-25 (Jaton, 1975). This peptide comprises the third hypervariable section of the H chain (residues 94–102).

Characterization of CNBr fraction I

The peptide comprised in this fraction was subjected to 29 cycles of automated Edman degradation. A single sequence could be determined (Fig. 5b), which was shown to be identical with that of the *N*-terminal region of the constant domain C_{H1} (Pratt & Mole,

1975; Jaton, 1975). Fraction I therefore contains the C_{H1} domain of the H chain BS-1 (residues 103–253).

The primary structure of the V domain of type III anti-pneumococcal antibody BS-1 is presented in Fig. 6. The sequence of the V_H region comprises that of the CNBr fragments 1–78 (fraction II) and 79–102 (fraction III), whereas that of the V_L region is taken from previous work (Jaton, 1974a).

Discussion

The data described here complete the structural analysis of the Fv domain of antibody BS-1, which was raised in an outbred rabbit against type III pneumococcal vaccine in the same way as antibodies BS-5 and K-25, whose full V-domain sequences have been described (Jaton 1974c, 1975). The obvious question to be asked is how the three antibodies BS-5, K-25 and BS-1 compare with each other in the hypervariable regions (Wu & Kabat, 1970) known to be directly involved in determining the antigen-binding specificity of the antibody (Poljak, 1975; Davies *et al.*, 1975). Is there any correlation between primary structure and antigen-binding specificity? Fig. 7 summarizes the amino acid sequences of the antibodies BS-1, BS-5 and K-25 in the first, second and third hypervariable segments of the H-chain V region. The fragmentary sequence information on three other antibodies to type III pneumococcal polysaccharide, designated K-17, K-325 (Strosberg *et al.*, 1972) and 3381-2 (Haber *et al.*, 1975), is also included.

In the first hypervariable zone spanning residues 29–36, serine-29 and tyrosine-31 are conserved residues, whereas variability is seen in positions 30, 32, 33 and 34. At these positions, polar and non-polar residues are being replaced by other polar and non-polar amino acids respectively (Fig. 7).

In the second hypervariable zone (residues 48–62), positions 49–53, 56 and 60–61 are variable. The remainder of the section appears invariant. Any two pneumococcal specific antibodies vary in at least two to six positions in that region: e.g. antibodies BS-5 and K-25 differ from each other by two residues, whereas antibodies 3381-2 and K-325 differ in at least six positions.

The section that encompasses positions 94 to about 108 is homologous to the fourth hypervariable region of the human H chains identified by Kehoe & Capra (1971); each of the four antibodies for which sequence information is available in that region exhibits a unique length that varies from nine amino acids in antibody BS-1 to 14 amino acids in antibody K-17, the longest hypervariable region thus far determined in a rabbit antibody. In that section, when the four antibodies are aligned for maximal homology, there is not a single position where the same amino acid residue is observed.

	30	35	50	60
BS-1	Ser-Ser-Tyr-Thr-Val-Ala-Trp-Val	Gly-Ile-Leu-Lys-Ser-Ser-Gly-Ser-Thr-Tyr-Tyr-Ala-Ser-Trp-Ala		
BS-5	Ser-Ser-Tyr-Asp-Met-Gly-Trp-Val	Gly-Ile-Ile-Tyr-Ala-Ser-Gly-Ser-Thr-Tyr-Tyr-Ala-Ser-Trp-Ala		
K-25	Ser-Gly-Tyr-Asp-Met-Ser-Trp-Val	Gly-Val-Ile-Tyr-Ala-Ser-Gly-Ser-Thr-Tyr-Tyr-Ala-Thr-Trp-Ala		
3381-2	Ser-Ser-Tyr-Ser-Met-Ala-Trp-Val	Gly-Val-Ile- ? -Ser-Gly-Gly-Ser-Ala-Tyr-Tyr-Ala-Ser-Trp-Ala		
K-325	ND	Gly-Ile-Ile-Tyr-Ala-Ser- ? -Ser-Pro-Tyr-Tyr-Ala-Gln-Ser-Ala		
Variability	V V V V V V V V V V V V V V V V			
	95	100	105	110
BS-1	Cys-Ala-Arg-Ile-Thr-His-Gly	--- Tyr-Leu-Gly-Leu --- Met ---	--- Asp-Val-Trp-Gly-Pro	
BS-5	Cys-Ala-Arg-Gln-Gly-Thr-Gly-Leu-Val-His-Leu-Ala-Phe	--- Val ---	--- Asp-Val-Trp-Gly-Pro	
K-25	Cys-Ala-Arg-Gly-His-Thr-Gly-Leu-Ser-Tyr-Leu-Lys-Ser-Ser-Val	---	--- Asp-Val-Trp-Gly-Pro	
K-17	Cys-Ala-Arg-Ser-Ile- ? -Thr- ? -Ile-Asp-Ser-Ser- ? ?	? -Phe-Leu-Asp-Val-Trp-Gly-Pro		
Variability	V V V V V V V V V V V V V V V V			

Fig. 7. Sequences in the hypervariable regions of rabbit anti-(type III) pneumococcal H chains

The sequences spanning residues 30-35, 50-60 and 94-110 are homologous to the first, second and fourth hypervariable regions respectively in human H chains (Capra & Kehoe, 1975). Sequence data on antibodies BS-1, BS-5 and K-25 are from Jatón (1974c, 1975; the present paper), the partial sequences of antibodies K-17 and 325 from Strosberg *et al.* (1972) and that of antibody 3381-2, from Haber *et al.* (1975). ND, Not determined. ?, Undetermined residue.

The examination of these three hypervariable sections suggests an apparent lack of sequence homology between the three anti-(type III) pneumococcal antibodies; this great sequence diversity in molecules endowed with the same antigen-binding specificity is particularly striking in the third hypervariable section of the V_H domain. As with the H chains, the V_L regions of antibodies BS-1, BS-5 and K-25 differ markedly in the third hypervariable zone spanning residues 87-97, whereas they are nearly identical in the second hypervariable section. This finding is consistent with additional sequence data on two rabbit anti-(pneumococcal L chains) (Margolies *et al.*, 1975). As inferred from X-ray-crystallographic studies of myeloma proteins, the pattern of insertions and/or deletions, which is characteristic of all hypervariable sections, seems to be the major factor which influences the shape of the binding site and which controls the specificity of the antibody (Poljak, 1975; Davies *et al.*, 1975). In view of the chemical simplicity of the antigenic determinant, cellobiuronic acid (Heidelberger, 1973), the absence of a correlation between primary structure and antigen-binding specificity in rabbit anti-SIII antibodies is surprising; for SIII antigen is a linear polymer of glucuronic acid (GlcA) and glucose (Glc) engaged in alternating internal β -1,4 and β -1,3 glycosidic linkages:

The hexasaccharide A has, for example, three β -1,4 linkages and two β -1,3 linkages, whereas the contiguous hexasaccharide B has three β -1,3 linkages and two β -1,4 linkages. SIII antigen may therefore contain at least two sets of overlapping determinants. GlcA β 1 $\rightarrow$ 4Glc and Glc β 1 $\rightarrow$ 3GlcA, because both may be considered as a repeating unit. Receptors on precursor cells in the rabbit may have the capability of preferentially recognizing either one of these linkages. Serological evidence for this has been reported in a study by Mage & Kabat (1963). In addition to this heterogeneity of specificity, the size of the antibody-combining site may be variable as evidenced by the observations of Mage & Kabat (1963) and of Campbell & Pappenheimer (1966), who found that small oligosaccharides produced varying degrees of inhibition in different rabbit antisera; this suggests that the sizes of the hypervariable segments are variable, an observation con-

sistent with the different length of the third V_H hypervariable regions of antibodies BS-1, K-25, BS-5 and K-17.

The association constant K_A of antibodies BS-1, BS-5 and K-25 for the hexasaccharide isolated from acid hydrolysis was shown to vary between 2.5×10^5 and $1.4 \times 10^6 \text{ M}^{-1}$ (Huser *et al.*, 1975). The analysis of the fine specificity by the oligosaccharide-inhibition technique will probably reveal structural differences between these proteins, yet a detailed knowledge of the specificity may be difficult to gain. Moreover, idiotypic determinants, shown to be closely associated with the hypervariable regions (Capra & Kehoe, 1974; Capra *et al.*, 1975a,b) and with the structure of the antigen-binding site (Brient & Nisonoff, 1970; Weigert *et al.*, 1974), are different for each of these three antibodies (Huser *et al.*, 1975). Thus one would expect that the anti-pneumococcal antibodies BS-1, BS-5 and K-25 would display distinctive sequences in the hypervariable regions.

Although structural data are as yet sparse, the diversity in sequences of hypervariable regions involved in the binding of type-III pneumococcal polysaccharide seems much larger than that seen in studying the structural and binding characteristics of mouse myeloma proteins with anti-hapten activity (Barstad *et al.*, 1974b; Claflin & Davie, 1974). In the latter case, proteins with shared idiotypic deter-

minants were identical through the first hypervariable region of their L and H chains (Barstad *et al.*, 1974a). This discrepancy between the complexity of the immune response to a simple linear polysaccharide antigen like SIII in the rabbit and the limited diversity of mouse myeloma proteins may be related to several not readily controllable factors, such as the heterogeneity of the determinants of the antigen (e.g. GlcA β 1 $\rightarrow$ 4Glc or Glc β 1 $\rightarrow$ 3GlcA in the SIII antigen), the nature of the selection process and the inbreeding of the animals. The origin of the V-region diversity, whether carried entirely in the genome (Dreyer & Bennett, 1965) or somatically generated from a small number of V genes (Jerne, 1971) still remains a controversial issue; it seems that, on the basis of sequence analysis of myeloma proteins and idiotypic analysis, there is good evidence for repeated V-region genes in inbred systems (Weigert *et al.*, 1970; Schlossman & Williamson, 1972). In outbred

populations, support for the stability of V-region genes is also provided by the suggestion that antibodies with identical combining sites have been elicited in two outbred rabbits (Montgomery *et al.*, 1975). Immunization of selectively bred rabbits with streptococcal vaccines provides evidence for the selection of antibodies with overlapping isoelectric-focusing patterns and cross-idiotypic specificities. This was not found in outbred rabbits [for a review, see, e.g., Braun & Jaton (1974)]. Further support for the concept that rabbits with related genetic background are capable of making similar antibodies comes from the observation that the sequence of six anti-streptococcal L chains was identical in the N-terminal 41 positions (Braun *et al.*, 1975). These data suggest that much of the genetic information necessary to account for V-region diversity is present and stably transmitted in the germ line. The selectively bred rabbit system may facilitate the identification of the structural correlate of the antigen-binding specificity because of the greater sequence uniformity than that of antibodies from outbred rabbits. However, the V-gene repertoire in inbred strains may not represent the full extent of diversity of V-region sequences, selected in outbred animals to fit the complementary structure of an antigen.

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