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Amino Acid Sequence of Rabbit Light Chains: Variable Region of a Light Chain from a Homogeneous Immunoglobulin Raised by Streptococcal Immunization†

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ABSTRACT: The variable region sequence has been determined for the light chain (L) from a rabbit homogeneous immunoglobulin (3547) produced by immunization with group A streptococcal vaccine. Unlike most immunoglobulins produced by these vaccines, this immunoglobulin had no binding activity for the group A polysaccharide nor for any of a wide range of streptococcal cell components tested, nor did it have binding activity for rabbit IgG. Tryptic digestion of the citraconylated L chain and acid hydrolysis of the aspartyl-proline bond at positions 109–110 were used to obtain two variable region peptides comprising residues 1–61 and 62–109, respectively. Automated sequence analysis of these peptides and the peptides obtained from them by complete tryptic digestion gave sequence data for the entire L-chain variable (V) region. Comparison of the 3547 L chain V region sequence with other data supports the observations that only two hypervariable regions are present in rabbit κ chains and that the hypervariable region beginning at residue 90 may vary in length by as much as six residues.

Rabbit homogeneous antibodies induced by immunization with various bacteria are currently employed in chemical studies to explore the structure-function relationships of antibodies. Allotypic and idiotypic markers of homogeneous antibodies induced in genetically defined animals are also used to study the genetic basis of antibody diversity (Kindt et al., 1974). Extensive sequence data on homogeneous antibodies are needed, however, to unravel the complexity of the antigen binding site and to reveal the precise structures for the various genetic markers. Sequence data are accumulating for rabbit antibody light (L^1) chains directed against *p*-azobenzoate (Appella et al., 1973) and polysaccharides from the group C streptococcus (Chen et al., 1974), the type 3 pneumococcus (Jaton, 1974a,b, 1975), and the type 8 pneumococcus (Margolies et al., 1975).

An unexpected feature of rabbit antibody V_L region structure is the presence of only two hypervariable regions. Although three hypervariable regions have been described for mouse and human κ chains (Wu and Kabat, 1970), only the first and third are present in the rabbit L chains. Another interesting aspect of rabbit antibody light chains concerns the high degree of variability observed among the N-terminal residues. Variation in this region has been used to divide V regions of κ chains into subgroups (Hood et al., 1970). Recent studies on N-terminal hypervariability suggest there are a larger number of subgroups than were previously estimated (Thunberg and Kindt, 1975). More sequence information will be required to define the variable region subgroups and to resolve questions concerning the relationship between variable region sequences and the group b allotypes of the constant region of the rabbit κ chain.

The present report describes sequence analysis of the V region of an allotype b4 L chain from a homogeneous immunoglobulin produced by a rabbit hyperimmunized with group A streptococci. While most homogeneous immunoglobulins produced in this fashion have antibody activity for the group A carbohydrate, homogeneous components for which no antibody activity can be detected have been observed in concentrations as high as 40 mg/ml. The immunoglobulin studied here is such an example. The strategy for the sequence determination relied heavily on automated sequence analysis of peptides obtained by tryptic digestions carried out after various chemical modifications of the L chain.

Materials and Methods

Isolation of 3547 L Chains. An immunoglobulin was isolated from the serum of rabbit 3547 by chromatography on DEAE-cellulose (Kindt et al., 1972). This isolated IgG was homogeneous by cellulose acetate electrophoresis and by alkaline urea disc electrophoresis of the L chains (Reisfeld

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[1] Abbreviations used are: Pth, phenylthiohydantoin derivative of an amino acid; SPITC, 4-sulphophenyl isothiocyanate; DMAA, dimethylallylaminetrifluoroacetic acid buffer, pH 9.5; AECys, *S*-2-aminoethyl-L-cysteine; H chain, heavy chain; L chain, light chain; V region, variable region; TC and TA, peptides from tryptic digests for the carboxamidomethylated and aminoethylated L chains, respectively; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

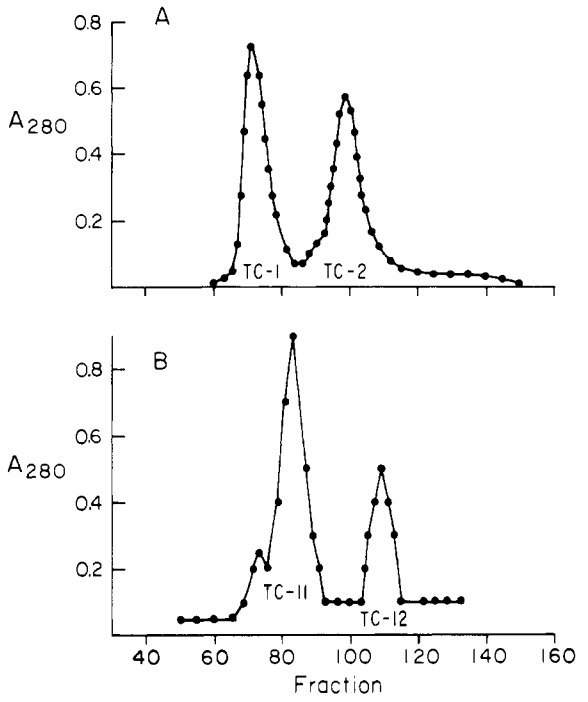

FIGURE 1: (A) Sephadex G-75 gel filtration in 5 M guanidine hydrochloride of tryptic peptides obtained by digestion of 40 mg completely reduced, carboxamidomethylated and citraconylated 3547 L chain. Peptides designated TC-1 and TC-2 span residues 62–209 and 1–61, respectively. (B) Sephadex G-75 gel filtration in 5 M guanidine hydrochloride peptide TC-1 (~6 mg) following acid cleavage of the aspartyl-proline bond at positions 109–110. Peptides designated TC-11 and TC-12 span residues 110–209 and 62–109, respectively.

and Small, 1966). The L chains were shown to be of the b4 allotype by quantitative serologic techniques (Kindt et al., 1972). H and L chains were prepared from 450 mg of IgG according to the principles of Fleischman et al. (1963). The L chains (120 mg) were lyophilized and stored at −20 °C.

Tryptic Digestion of Reduced L Chains. Isolated L chains (40–60 mg) suspended in 7 M guanidine hydrochloride (0.2 M Tris, pH 8.2) were reduced completely by addition of solid dithioerythritol to a concentration of 0.02 M. After incubation at ambient temperature for 1 h under N_2 in the dark, iodoacetamide-*2*-^{14}C (50 μCi) was added to the reaction mixture. Five minutes later, solid iodoacetamide was added to a concentration of 0.042 M. After 20 min, the mixture was dialyzed against N_2 saturated distilled H_2O at 4 °C in the dark.

As an alternative to carboxamidomethylation, completely reduced L chains were reacted with ethylenimine (2.5-fold molar excess over dithioerythritol) to convert cysteine residues to aminoethylcysteine (Slobin and Singer, 1968).

Prior to digestion of the L chain with trypsin, the primary amino groups were blocked by reaction with citraconic anhydride (Dixon and Perham, 1968). The reduced and alkylated (or aminoethylated) L chain (10 mg/ml) was suspended in 0.02 M borate buffer, pH 8.1, and reacted with citraconic anhydride (20-fold molar excess over lysine plus aminoethylcysteine). Dilute NaOH (0.1 M) was added to maintain pH 8. The L chains were lyophilized after exhaustive dialysis against dilute NH_4OH (0.001 M).

The completely reduced, alkylated and citraconylated L chains were digested with 1% (w/w) TPCK trypsin (Worthington Biochemicals) in 0.02 M ammonium bicarbonate buffer, pH 8.2, at 37 °C for 4 h. Identical tryptic digestion procedures were used for citraconylated L chains that were carboxamidomethylated or aminoethylated.

Peptides from the tryptic digests of the citraconylated L chains were isolated by gel filtration on a column of Sephadex G-75 (2.9 × 130 cm) in 5 M guanidine hydrochloride brought to pH 8.0 by the addition of solid Tris base. The separated peptides were dialyzed against distilled H_2O in dialysis tubing with a 3500 dalton molecular weight inclusion (Spectrum Medical Corp., New York) and lyophilized. The larger of the peptides from these digests was acid-cleaved at a susceptible aspartyl-proline bond (positions 109–110) by incubation at pH 2.5 for 5 days, according to the method described by Fraser et al. (1972). Peptides obtained from the acid cleavage were separated on Sephadex G-75 and then dialyzed and lyophilized as above.

The citraconyl groups were removed from peptides by incubation in 10% (v/v) acetic acid at 37 °C for 4 h. Following lyophilization, aliquots of peptides were again digested with 2% (w/w) TPCK trypsin at 37 °C for 2 h in 0.02 M ammonium bicarbonate buffer, pH 8.2. Peptides resulting from these second tryptic digests were separated on a column (0.9 × 10 cm) of Technicon Chromobeads Type P in pyridine formate buffer at 52 °C. A gradient (500 ml) extending from 0.2 N pyridine formate, pH 3.0, to 2.0 N pyridine formate, pH 5.0, was used. Peptides were detected in the eluted fractions with fluorescamine (Roche) and quantitated in a filter fluorometer (GK Turner and Assoc.)

Compositional and Sequence Analysis of Tryptic Peptides. Peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h in vacuo (Moore and Stein, 1963) and analyzed on a Durrum D500 amino acid analyzer. Cysteine was determined as carboxymethylcysteine or aminoethylcysteine.

Automated sequence analysis was carried out on 100–300 nmol of peptide on a Beckman 890B sequencer using an improved DMAA program (Beckman peptide program No. 111374. Tryptic peptides with C-terminal lysine were treated with SPITC (4-sulphophenyl isothiocyanate) to prevent their loss during automated sequencing (Inman et al., 1972). The recovered thiazolinones were converted to Pth-amino acids and identified by: (1) gas chromatography in a Beckman GC45 on SP 400 (Pizano and Bronzert, 1960); (2) thin-layer chromatography on polyamide sheets according to the methods of Summers et al. (1973); and (3) amino acid analysis on the Durrum D500 analyzer of the free amino acids recovered by back-hydrolysis of the Pth derivatives with HI (Smithies et al., 1971). Cysteine was determined as the [^{14}C]carboxamidomethyl derivative by counting in a liquid scintillation counter a 10% aliquot of the Pth derivative at each step.

Amino terminal residues of peptides were determined by a modification of the subtractive Edman degradation as described by Salnikow et al. (1973).

Carboxyl terminal residues were determined by amino acid analysis subsequent to digestion with carboxypeptidase B according to the method of Ambler (1967).

Results

Large peptides were obtained by tryptic digestion of the L chain after the lysine and aminoethylcysteine residues had been blocked by reaction with citraconic anhydride. Because L chain 3547 had two arginine residues, these tryptic digests resulted in only three peptides. A first tryptic digest was performed on 40 mg of citraconylated, carboxamidomethylated L chain; a second on 60 mg of citraconylated,

Table I: Amino Acid Compositions of Variable Region Tryptic Peptides.[a]

Amino Acid	Peptide							
	TA-2	TA-22	TA-27	TA-25	TC-12	TA-127	TA-125	TA-124
CMCys[c]					1.3 (2)			
Asp	4.8 (5)	2.2 (2)	2.3 (2)	1.2 (1)	3.9 (3)		1.2 (1)	0.9 (1)
Thr	5.4 (4)	3.7 (4)			5.6 (6)		1.2 (1)	2.4 (2)
Ser	7.6 (8)	3.0 (3)	2.2 (2)	2.7 (3)	5.5 (6)			2.6 (3)
Glu	6.2 (6)	1.2 (1)	4.6 (5)		4.4 (4)			2.1 (2)
Pro	4.1 (5)	0.5 (1)	2.9 (3)	0.9 (1)				
Gly	4.8 (4)	2.3 (2)	1.1 (1)	1.2 (1)	8.0 (9)			3.7 (4)
Ala	8.4 (9)	3.2 (3)	3.4 (3)	3.2 (3)	5.2 (5)		2.7 (3)	1.7 (2)
Val	4.6 (4)	3.4 (3)		1.1 (1)	5.6 (5)			2.8 (4)
Met	0.9 (1)	1.0 (1)						
Ile	2.9 (3)	1.1 (1)	1.0 (1)	1.0 (1)	1.3 (1)			
Leu	4.0 (4)		1.3 (1)	2.8 (3)	1.4 (1)			
Tyr	3.0 (3)	1.1 (1)	1.3 (1)	1.1 (1)	3.4 (4)		1.7 (2)	1.1 (1)
Phe					2.2 (2)	1.0 (1)		0.9 (1)
His								
AECys	1.0 (1)	0.9 (1)					0.9 (1)	
Lys	1.9 (2)		1.7 (2)		1.8 (2)	1.0 (1)		0.8 (1)
Arg	0.8 (1)			0.9 (1)				
Trp	(1)[b]		(1)[b]					
No. of residues	61	23	22	16	50	2	8	21
Residue position	1–61	1–23	24–45	46–61	62–109	62–63	81–88	89–107

[a] Values are given in residues per molecule. Values calculated from sequence analysis are given in parentheses. TA refers to a peptide recovered from the digest of citraconylated aminoethylated L chain. TC refers to a peptide recovered from the digest of citraconylated carboxamidomethylated L chain. [b] Presence of one residue of tryptophan assumed from sequence analysis of whole L chain and from sequence analysis of peptide T-27. [c] Carboxymethylcysteine.

aminoethylated L chain. Peptides from these digests will be designated TC for the carboxamidomethylated and TA for the aminoethylated L chain, respectively.

Isolation of Variable Region Peptides. After tryptic digestion of the citraconylated, carboxamidomethylated L chain, two major peptides were isolated by gel filtration on Sephadex G-75 in 5 M guanidine hydrochloride. Figure 1A depicts the gel filtration profile of these peptides, designated TC-1 (residues 62–209) and TC-2 (residues 1–61). A third peptide which spans residues 210–212 was not isolated.

An aliquot (approximately 6 mg) of peptide TC-1 was cleaved at the acid labile aspartyl–proline bond (positions 109–110) as described by Fraser et al. (1972), and the resulting peptides were separated by gel filtration on Sephadex G-75. The separation of peptides from the acid cleavage of peptide TC-1 is depicted in Figure 1B. Peptide TC-11 accounts for resides 110–209; peptide TC-12 accounts for residues 62–109.

A second tryptic digest was performed on 60 mg of citraconylated, aminoethylated L chain and peptides TA-1 (35 mg) and TA-2 (15 mg) were isolated as described above. Acid cleavage of peptide TA-1 gave unexpectedly low yields of peptide TA-11 (40%) and peptide TA-12 (50%, profile not shown). Attempts to acid-cleave certain other aminoethylated L chains have met with similar difficulties (A. Thunberg and R. Kutny, unpublished data).

Peptides TA-2 and TA-12 were digested with trypsin after the citraconyl groups had been removed. The resulting peptides were fractionated by chromatography on Technicon Chromobeads Type P in pyridine formate buffer. Peptides TA-22, TA-25, and TA-27 accounted for the entire sequence of peptide TA-2. Peptides TA-124, TA-125, and TA-127 accounted for the sequence of peptide TA-12 with the exceptions of positions 64–80 and positions 108–109. Compositions for the peptides used in the sequence determination are given in Table I. The composition of peptide TC-12, from the digest of the carboxamidomethylated L chain, is given in place of the composition of peptide TA-12.

Sequence Analysis of Whole L Chain. Intact carboxamidomethylated L chain was subjected to automated Edman degradation. Identifications were made for 48 of the first 49 residues (Table II). Residue 34 was identified as either Ala or Ser by back-hydrolysis with HI.

Analysis of Peptides from Digest of Carboxamidomethylated L Chain. Peptide TC-1 (Positions 62–209). Automated Edman degradation through the amino-terminal 17 steps was performed on peptide TC-1. The sequence is shown in Table II.

Peptide TC-12 (Positions 62–109). Peptide TC-12 was reacted with SPITC and 29 cycles of automated Edman degradation were performed. The N-terminal Phe and the Lys at step 2 were not identified because their free amino groups had reacted with SPITC. Step 15 was lost. The sequence of the first 29 residues is given in Table II.

Analysis of Peptides from Digest of Aminoethylated L Chain. Peptide TA-22 (Positions 1–23). Peptide TA-22 was not sequenced. Its composition matches that expected for the N-terminal 23 residues of the L chain (Table I). Peptide TA-22 contained the only methionine residue (position 4) in L chain 3547.

Peptide TA-27 (Positions 24–45). This peptide was treated with SPITC prior to sequence analysis. Peptide TA-27 contained an internal lysine residue in addition to that at the C terminus. The internal lysine was adjacent to proline and therefore resistant to tryptic digestion. The C-terminal lysine was not released by digestion with carboxypeptidase B, probably because two proline residues were present in subterminal positions. The amino terminal sequence, Gln-Ala, was identified by sequence analysis of a sample of pep-

Table II: Sequence Analysis of Peptides.[a]

Peptides	Residue Positions	
L chain	1–212	1 10 20 30 A Y D M T Q T P S S V S A A V G G T V T I N C Q A S E D I S → 31 40 49 A N L A/S W Y Q Q K P G Q P P K L L I Y → → → → → → → → → → → → → → → → → → →
TA-27	24–45	24 30 40 45 Q A S E D I S A N L A W Y Q Q – P G Q P P – a → → → → → → → → → → → → → → a → → → → → a → →
TA-25	46–61	46 50 60 L L I Y A A S D L A S G V P S R → → → → → → → → → → → → → → → → ←
TC-1	62–209	62 70 78 F K G S G S G T E Y T L T I S G V → → → → → → → → → → → → → → → → →
TC-12	62–109	62 70 80 90 – – G S G S G T E Y T L T I – G V Q C A D A A T Y Y C Z S a a → → → → → → → → → → → → b → → → → → → → → → → → → → →
TA-125	81–88	81 88 A D A A T Y Y C a → → → → → → a → ←
TA-124	89–107	8990 97 a b98 100 107 Q S A D Y S G S A V T F G G G T E V V V K a → → → → → → → → → → → → → → → → → → → a → → → ←

[a] Symbols have the following meanings: (⇀) analysis by automated sequencer; (→) analysis by manual Edman or by repeat sequencer run on material not reacted with SPITC; (←) analysis by carboxypeptidase B; (a) residue not identified during automated sequence analysis because its free amino group had reacted with SPITC; (b) sample lost.

tide TA-127 not treated with SPITC. The sequence of peptide TA-27 is given in Table II.

Peptide TA-25 (Positions 46–61). The sequence of peptide TA-25, determined by automated Edman degradation, is given in Table II. The C-terminal arginine was identified both by back-hydrolysis of the Pth derivative and by digestion with carboxypeptidase B.

Peptide TA-127 (Positions 62–63). Peptide TA-127 was not sequenced. The composition (Table I) corresponds to the N-terminal two residues of peptide TA-12.

Peptide TA-125 (Positions 81–88). Peptide TA-125 was reacted with SPITC and sequenced in the Beckman sequencer. Step 1 was identified by manual Edman degradation as alanine; step 8 was identified by carboxypeptidase B digestion as aminoethylcysteine. The sequence of peptide TA-125 is given in Table II.

Peptide TA-124 (Positions 89–109). Peptide TA-124 was reacted with SPITC and sequenced in the Beckman sequencer. C-terminal lysine was identified following digestion with carboxypeptidase B. A second sequence analysis on a small amount of underivatized peptide gave Gln-Ser-Ala as the N-terminal sequence. The sequence of peptide TA-124 is given in Table II.

Summary of Sequence Information. Figure 2 summarizes the sequence information obtained from the tryptic peptides from the aminoethylated (TA) and carboxamidomethylated (TC) 3547 L chain. These peptides account for the entire V region of L chain 3547. Overlapping peptides have been obtained in all instances except for positions 61–62. Despite the absence of this one overlap, the alignment of the peptides proposed in Figure 2 is supported by sequence information obtained on other b4 L chain V regions (Appella et al., 1973; Chen et al., 1974; Jaton, 1974a,b, 1975; Margolies et al., 1975). Figure 3 compares the V region sequence of L chain 3547 with the V region sequences of several other allotype b4 L chains.

Discussion

The amino acid sequence of the variable region of a rabbit immunoglobulin L chain was determined by analysis of peptides from tryptic digests of the L chain. Initial digests were limited to arginine residues by blocking lysine and aminoethylcysteine residues with citraconic anhydride prior to tryptic digestion. These digests yielded two major peptides spanning residues 1–61 and 62–209. The latter peptide was cleaved at an aspartyl-proline bond by reaction with dilute acid (Fraser et al., 1972), yielding two smaller peptides spanning residues 62–109 and 110–209. The entire variable region was therefore present in two peptides (1–61 and 62–109). These were subjected to tryptic cleavage at lysine and aminoethylcysteine residues following removal of the citraconyl groups. Analyses of these peptides yielded a V region sequence complete with exception of a single overlap between residues 61 and 62. Alignment of peptides proposed for this region is in complete agreement with sequence data reported for other rabbit L chains. The success of this determination has provided a complete strategy for structure determinations on rabbit L chains from homogeneous antibodies using automated sequencer techniques.

The majority of rabbit antibodies elicited by group A streptococcal vaccine have activity for the streptococcal group specific carbohydrate or for some antigen on the cell surface (Krause, 1970). In certain instances, homogeneous antibodies with anti-IgG activity have been isolated from

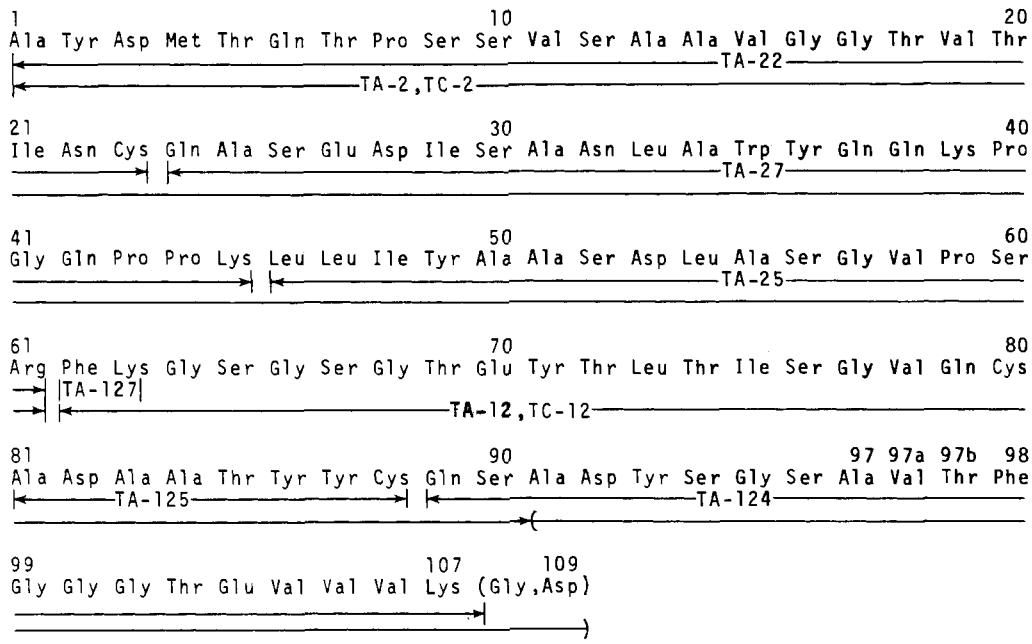

FIGURE 2: Summary of sequence data obtained for the V region of 3547 L chain. Insertions are introduced at position 97 to maximize homology to human κ light chains.

Amino Acid Sequences

L Chain	0 1	10	20	30	32 a b
3547	[]A Y D M T Q T P S S V S A A V G G T V T I N C Q A S E D I S A N [][]L A W				
4135	A D I V——————A———E P——————K———T——Q S——D D Y [][]——S—				
3315	A Q I V——————————————————S——Q S V Y E——G R——S—				
BS-5	[]D V V——————A———E P——————K———Q S——Y S N [][]———				
2717	[]V E V L——————P——————S———S T K S——Y B B B Y———				

	36	46	56	66	73
3547	Y Q Q K P G Q P P K L L I Y A A S D L A S G V P S R F K G S G S G T E Y T L				
4135	——————G———R——T——————R——————D F——				
3315	F——————R———R——T——————S———T——————Q F——				
BS-5	——————K———T——E——————————————D F——				
2717	——Z——————A———T——S——————T——————Z F——				

	74	84	97 a b c d	100	107
3547	T I S G V Q C A D A A T Y Y C Q S A D Y S G S A V T [][]F G G G T E V V V K				
4135	———D L E——————————T Y G V——[][][][][][] ——————				
3315	S———D———D——————L G N Y D C S——G D S F T ——————				
BS-5	———D L E——————F———G S B——T——T V [][][][] ——————				
2717	—L——D——Z——D——————G G———T——Y S [][][][] ——————				

FIGURE 3: Comparison of 3547 V region sequence to other b4 L chains raised by immunization with various antigens. Brackets ([ ]) represent insertions introduced to maximize homology among the sequences. 4135 (Chen et al., 1974): anti-group C streptococcal polysaccharide. 3315 (Margolies et al., 1975): anti-SVIII pneumococcal polysaccharide. BS-5 (Jaton, 1974b): anti-SIII pneumococcal polysaccharide. 2717 (Appella et al., 1973): anti-*p*-azobenzoate.

the sera of rabbits hyperimmunized with streptococci (Bokisch et al., 1972). The antibody studied here had no activity toward any of the antigens mentioned above, nor would it react with the whole vaccine against which the animal was immunized. Neither the intact immunoglobulin nor the purified H and L chains showed any irregularity in physical or chemical property (A. Thunberg unpublished data). It is puzzling that an antibody with no detectable binding capacity can be produced in such high concentration.

Shown in Figure 3 are the amino acid sequence of the variable region of L chain 3547 and the V region sequences of several other allotype b4 L chains of different antigen binding specificities. This comparison reveals several features of rabbit κ chain V regions that make them quite different from those of the mouse and human. First, the L chains are of different lengths at the N terminus when aligned for maximum homology. This difference in length has been used to define V_κ subgroups (Hood et al., 1970). By this definition, L chains 3547, BS-5, and 2717 are of the $V_{\kappa II}$ length and L chains 4135 and 3315 are of the $V_{\kappa I}$ length. L chains with the amino terminal residue at position 2 are in the subgroup $V_{\kappa III}$. It has previously been pointed out that the differences in the length and in the amino acid at the N terminus are inadequate to accurately define V_κ subgroups (Thunberg and Kindt, 1975). The complete V region sequences obtained to date, however, have not provided sufficient information to improve on the current definition. For example, differences in the length of the hypervariable regions are not readily correlated with the N-terminal differences (Figure 3). There is no evidence to suggest that the variability within the amino-terminal four residues is associated with the binding site.

Regions of striking variability are present at positions 30–32b and 89–97d. These hypervariable regions are proba-

bly binding-site related, forming a part of the antigen binding cleft of the antibody (Poljak, 1975). Unlike human and mouse L chains, there does not seem to be an additional hypervariable region between residues 45 and 64. The sequences of this region for the rabbit L chain are quite constant, with the exception of certain positions (46, 50, 53, and 63), which are variable. It is likely that the rabbit L chain has only two hypervariable regions (Margolies et al., 1975; Poljak, 1975).

While considerable information on the structure of rabbit L chains is now available, more data must be obtained. Sequences of L chains of all group b allotypes with a variety of binding specificities are required to define the relationship of sequence to binding specificity, to determine the nature of the V region subgroups, and to establish the relationship (if any) between V region sequence and the group b allotypes of the C region (Hood et al., 1971; Thunberg et al., 1973; Thunberg and Kindt, 1975). In addition, data on L chains from genetically defined antibodies of closely related family members will be required to establish relationships between idiotypes and hypervariable region sequence, and to begin to explore questions on the genetic control of antibody synthesis (Kindt et al., 1974).

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