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Sequence of a Rabbit Anti-*Micrococcus lysodeikticus* Antibody Light Chain†

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ABSTRACT: The complete sequence of rabbit antibody light-chain L 120 has been elucidated. The antibody was raised against *Micrococcus lysodeikticus* bacteria and is specific for the external part of the cell wall. All protein used in this work was obtained from a single 50-mL bleeding. The variable region of L 120 is compared to 13 other sequences of chains of different specificities. The constant region of this b4 κ chain is identical to that of two other constant regions published earlier. The general structure of the rabbit light chain is compatible with the three-dimensional folding proposed for human myeloma chains.

The elucidation of the structure-function relationship and of the genetic mechanisms involved in IgG synthesis relies on the comparison of the sequences of a large number of light and heavy chains from naturally raised antibodies for which the specificity is well established. While many myeloma human and mouse immunoglobulin antibody sequences have been reported, only a few primary structures of induced rabbit antibody molecules are available; mainly those of anti-pneumococcus type III and type VIII, anti-streptococcus antibodies (Chen et al., 1974; Jaton, 1974a,b, 1975; Margolies et al., 1975; Braun et al., 1976), and anti-*p*-azabenzoarsonate antibodies (Appella et al., 1973).

We report here the complete light-chain sequence of a rabbit antibody raised against *Micrococcus lysodeikticus*, a gram-positive bacterium. This antibody is specific for the external part of the *Micrococcus lysodeikticus* cell wall, which is composed of a polymer of glucose-*N*-acetylmannoseaminuronic acid. A comparison of the L 120 sequence with previously reported anti-pneumococcal, anti-streptococcal and anti-*p*-azabenzoarsonate light chains is discussed, and the existence

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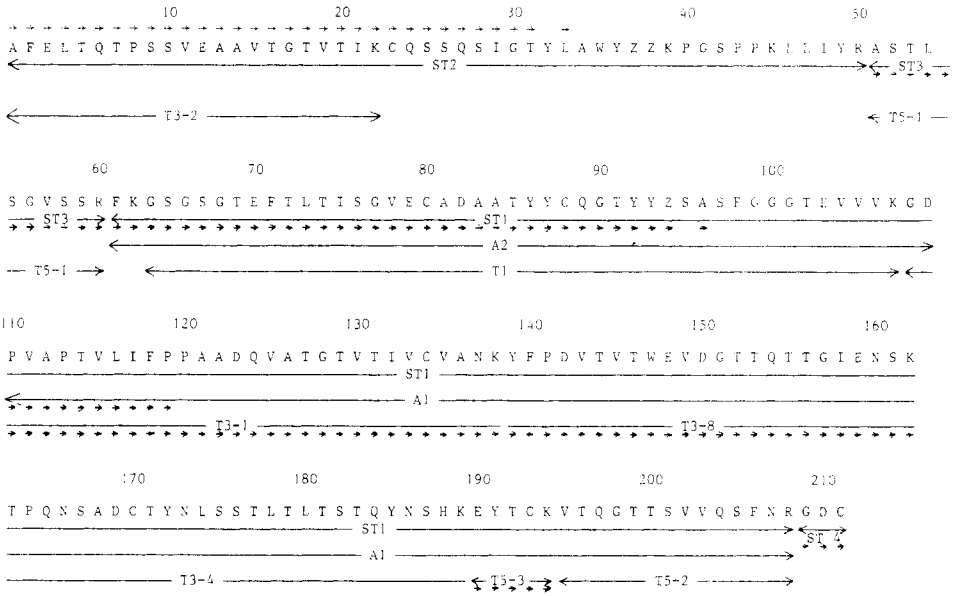

FIGURE 1: Sequence results obtained by direct Edman degradation of the whole light chain and by analysis of the acid cleavage fragments (A1 and A2) and the tryptic peptides of the succinylated and untreated light chain (T1 to T9) (ST1 to ST3). Residues underlined by arrows have been sequenced directly. In the chymotryptic peptides, other residues were either sequenced or assigned by homology (Figures 5 and 6).

and location of the hypervariable regions and framework residues are confirmed within the antibodies of this new specificity. The rabbit light-chain sequences obtained so far are compatible with the three-dimensional structure proposed for several myeloma light chains (Poljak et al., 1974; Schiffer et al., 1973; Segal et al., 1974).

Experimental Procedures

Preparation of Antibody and Light Chain 120. Rabbit antibody light chain 120 was immunized with *Micrococcus lysodeikticus* without interruption following a schedule of injections described previously (Van Hoegaerden et al., 1975). The antibody L 120 was isolated from serum dialyzed against 0.005 M phosphate (pH 7.2) by chromatography on DEAE-cellulose (DE 52 Whatman) equilibrated, using the same buffer. A single bleeding was used, and 2.2 g of homogeneous antibody was recovered from 50 mL of plasma. The specificity of the homogeneous antibody L 120 preparation was determined by inhibition of the quantitative polysaccharide precipitation with either pure peptidoglycan or pure carbohydrate polymer of the *Micrococcus lysodeikticus* cell wall (Wikler, 1976).

The immunoglobulin was subjected to mild reduction using 0.1 M 2-mercaptoethanol in 0.4 M Tris-HCl buffer (pH 8.2) at 37 °C for 90 min, followed by alkylation by the addition of a solution of iodoacetamide in the same buffer in 10% molar excess over the 2-mercaptoethanol. The latter reaction was allowed to proceed for 15 min at 4 °C. The reaction mixture was then dialyzed against 1 M acetic acid for 18 h, with three changes of dialysate (Fleischman et al., 1963). Light and heavy chains were separated by gel filtration on Sephadex G-100 (Pharmacia) in 1 N acetic acid according to Fleischman et al. (1963). Succinylation of the light chain was performed according to the procedure of Klotz (1967): Lyophilized light chain was dissolved in water and a tenfold molar excess of solid succinic anhydride with respect to lysine was added gradually over 1 h while the pH was maintained at 9.0 by the addition of 1 N NaOH.

Full reduction was carried out in 0.01 M dithiothreitol, 7 M guanidine hydrochloride, and 0.5 M Tris-HCl (pH 8.5) for 90 min at 37 °C. This was followed by the addition of iodoacetic acid in 10% molar excess over the dithiothreitol, and the reaction was allowed to proceed for 15 min at 4 °C. Iodo[2-^{14}C]acetic acid (Amersham, Searle Co.) was added to yield a specific activity of 2–4 μCi/mg light chain. Polyacrylamide disc gel electrophoresis was performed in 8 M urea at pH 9.5, as described previously (Pincus et al., 1970).

Hydrolytic Methods. Hydrolysis of fully reduced and alkylated light chains was performed with TPCK-treated trypsin (Worthington) in 1% ammonium bicarbonate (pH 8.2) at 37 °C. The initial substrate/enzyme ratio was 100:1. After 3 h, an equal amount of trypsin was added and the digestion was allowed to proceed for an additional 3 h.

Succinylated light chain was subjected to acid hydrolysis in 10% acetic acid–pyridine (pH 2.5) in 7 M guanidine hydrochloride, as described by Fraser et al. (1972).

Isolated peptides were digested with chymotrypsin, using a 100:1 peptide/enzyme ratio in 1% ammonium bicarbonate (pH 8.2) at 37 °C for 30 min.

Amino acid compositions were determined on a Durrum 500C analyzer. Hydrolysates were prepared employing 1 mL of constant-boiling HCl at 110 °C for 24 h in vacuum-sealed tubes.

Separation of Peptides. Tryptic and chymotryptic digests were resolved by gel filtration on Sephadex G-25 superfine and G-50 or G-100 in 0.05 M or 1 M NH_4OH. Isolated fractions were chromatographed on DEAE-Sephadex A-25 using linear gradients from 0.005 to 0.3 or 1 M NH_4HCO_3 (pH 8.5). High-voltage electrophoresis employing Whatman 3MM paper and pyridine–acetate buffers at pH 3.6 and 6.5 was also used for the purification of peptides, which were detected with the ninhydrin–cadmium staining of monitor strips and eluted using the electrophoresis buffer. Aliquots of all fractions were counted in Bray's solution in a Packard liquid scintillation counter.

Sequence Analysis. The intact light chain 120, the large fragments obtained by acid hydrolysis, and the tryptic peptides were sequenced using a protein sequenator (Beckman Sequencer 890C). The following programs were employed: 1 M Quadrol (Edman, 1970), dimethylallylamine (DMAA) (Capra & Kehoe, 1974), and 0.1 M Quadrol (Brauer et al., 1972).

TABLE I: Amino Acid Composition of L 120 and Tryptic Peptides of the Succinylated Chain.[a]

amino acid	L 120	ST2	ST3	ST1
CMCys	7	1.2	1.2	4.8
Asp	13	2.1	1.0	12.0
Thr	31	4.9	1.1	26.6
Ser	22	4.9	3.7	12.8
Glu	22	8.0		14.9
Pro	10	3.3		7.5
Gly	20	4.2	2.5	13.6
Ala	17	4.7	1.9	11.0
Val	20	3.0	1.0	15.5
Ile	7	1.8		2.9
Leu	10	3.6	1.1	4.6
Tyr	10	1.9		6.8
Phe	7	0.7		6
His	1			1.6
Lys	9	3.0		4.0
Arg	3	1.2	0.8	1
Trp[b]	2	1		1
total res	211	47.3	14.3	146.6

[a] Values are residues/molecule of peptide or protein. [b] As determined by sequence.

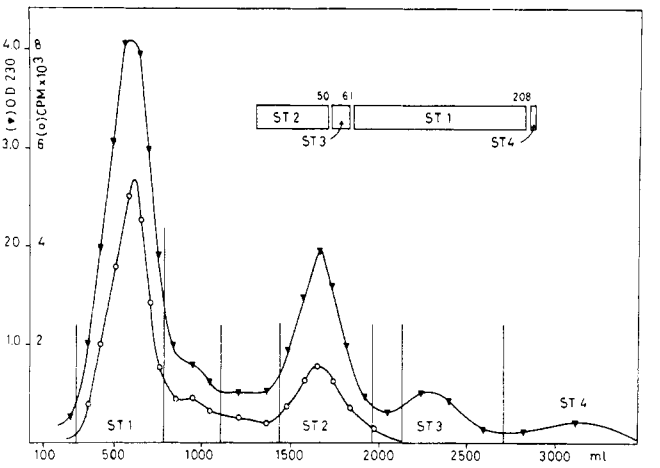

FIGURE 2: Separation of the peptides obtained after trypsinolysis of succinylated light chain. The column (2.5 × 200 cm) of Sephadex G-100 was developed in 1 N NH_4OH. The ordering of the peptides is represented schematically in the insert.

TABLE II: Automated Sequence Analysis of Peptide ST1.

step	GC	TLC	amino acid anal.[b]	cpm	res
1	F	F	F	245	Phe
2		K	K	245	Lys
3	G	G	G	125	Gly
4	S		A	320	Ser
5	G	G	G	275	Gly
6	S		A	300	Ser
7	G	G	G	380	Gly
8	T	T	α-AB[a]	300	Thr
9	E	E	Z	250	Glu
10	F	F	F	250	Phe
11	T		α-AB	190	Thr
12	L	L/I	L	250	Leu
13	T		α-AB	215	Thr
14	I	L/I	I	340	Ile
15	S		A	290	Ser
16		G	G	320	Gly
17	V	V	V	350	Val
18	E		Z	370	Glu
19		CMCys	A	2300	CMCys
20	A	A	A	1640	Ala
21	D	D	B	635	Asp
22	A	A	A	320	Ala
23	A	A	A	410	Ala
24	T	T	α-AB	390	Thr
25	Y	Y	Y	335	Tyr
26	Y	Y	Y	405	Tyr
27		CMCys	A	1580	CMCys
28		Q	Z	1110	Gln
29		G	G	605	Gly
30	T or S		α-AB	375	Thr
31	Y	Y	Y	335	Tyr
32	Y	Y	Y	270	Tyr
33	Q	Q		250	Gln
34				315	
35	A		A	270	Ala

[a] α-AB or α-aminobutyric acid is the breakdown product of Pth-Thr. [b] After HI back-hydrolysis.

The average repetitive yield in the sequenator ranged from 95 to 98%. Lysine-containing tryptic peptides were modified prior to sequence determination by the method of Braunitzer et al. (1972). The Pth derivatives of the amino acid residues were identified by gas–liquid chromatography (Pisano & Bronzert, 1969), by thin-layer chromatography on polyamide sheets (Summers et al., 1973), and by back-hydrolysis of Pth derivatives in HI at 150 °C in vacuum-sealed tubes, followed by amino acid analysis (Van Orden & Carpenter, 1964). Pth-*S*-[^{14}C[carboxymethylcysteine (SCMC) was estimated by counting of a 5% aliquot.

The small, chymotryptic peptides were sequenced using the dansyl-Edman procedure (Gray, 1967), supplemented by amino acid analysis of the HI hydrolysate of the removed residue at every step of the procedure.

Results

Homogeneity of Antibody 120. The criteria for homogeneity of antibody 120 used in this work were: single light- and heavy-chain bands on polyacrylamide gel electrophoresis (Van Hoegaerden et al., 1975) and a single amino acid residue at each of the 31 steps of the automated Edman degradation, starting at residue 1 of the N terminus of the light chain (Figure 1). The homogeneity of the heavy chain was also supported by partial sequence determination (Van Hoegaerden & Strosberg, 1976). Compositional analysis of fully reduced and alkylated L 120 is presented in Table I and shows the presence of 7-SCMCys, suggesting that this b4 κ chain contains three intrachain disulfide bonds in addition to one bond linking light and heavy chains.

Analysis of Peptides Obtained after Succinylation of L 120. The amino acid analysis of L 120 indicates that the chain contains only three arginine residues (Table I). The light chain was succinylated after full reduction and alkylation and submitted to tryptic digestion. The tryptic peptides were separated by gel filtration on Sephadex G-100 (Figure 2).

Several authors have reported the constant-region sequence of rabbit b4 light chains, indicating that only one arginine residue is present at position 208 of the chain (Strosberg et al., 1972; Appella et al., 1973; Chen et al., 1974; Margolies et al., 1975). The size of the peptides, their composition, and their partial or complete sequence indicate that the arginine residues of L 120 are at positions 50, 61, and 208, as represented in Figure 2. Compositions are presented in Table I and sequence data for peptides ST1 and ST3 are presented in Tables II and III, respectively. The amino acid analysis of ST3 indicates the presence of peptide ST4 (Table I). This tripeptide, therefore, coeluted with ST3, although it was also detected in the last

TABLE III: Automated Sequence Analysis of Peptide ST3.

step	GC	TLC	amino acid anal.	residue
1	A + G	A + G	A + G	Ala + Gly[a]
2	D + S	D + S	B + A	Ser + Asp[a]
3	T	SCMC	AB	Thr + Cys[a]
4	L	L/I	L	Leu
5	A	A	A	Ala
6			A	Ser
7	G		G	Gly
8	V	V	V	Val
9			A	Ser
10			A	Ser
11			R	Arg

[a] The sequence Gly-Aps-Cys corresponds to fraction ST4 and is present here as a contaminant of fraction ST3.

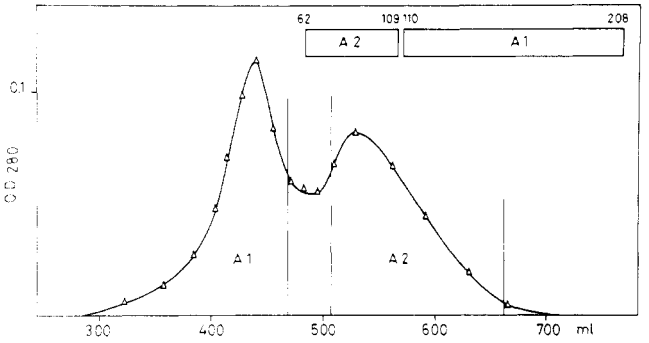

FIGURE 3: Separation of the fragments obtained by acid cleavage of peptide ST1. The column was the same as that described under Figure 2. The ordering of the fragments resulting from the hydrolysis of peptide bond Asp-109–Pro-110 is presented in the insert.

peak (Figure 2) which corresponds to the total volume of the column. The assignment of serine to positions 6, 9, and 10 was based on the absence of alanine on gas chromatographic analysis and on its presence after back-hydrolysis and amino acid analysis. The largest fragment obtained by trypsinolysis of the succinylated chain ST1 was further degraded by acid hydrolysis (Fraser et al., 1972), to specifically cleave the Asp–Pro bond located at positions 109 and 110 of the light chains of allotype b4. The digest was fractionated on Sephadex G-100, as shown in Figure 3, resulting in two fragments. The composition of the smaller fragment, A2, is presented in Table IV. Its N-terminal sequence corresponds to the part of the light chain starting at position 62. The larger fragment A1 was partially sequenced for ten residues to confirm its identity with that of the b4 constant region previously reported (Chen et al., 1974). This result is included in Figure 1, summarizing all the sequence determinations.

Analysis of Typtic Peptides from L 120. Tryptic peptides from fully reduced and alkylated L 120 were separated on Sephadex G-50 (Figure 4). The peptides were further purified by ion-exchange chromatography on DEAE-Sephadex A-25, as described under Experimental Procedures. The compositions of the purified tryptic peptides are given in Table IV and their sequences in Figure 1.

Analysis of Chymotryptic Digests of L 120 Tryptic Peptides. Peptide T1 extending from residue 64 to 107, as evidenced by its composition (Table IV), and the sequences of ST1 and A1, was digested with α-chymotrypsin, yielding four major and several minor peptides which were separated by high-voltage electrophoresis on paper in pyridine–acetate at pH 3.5. The compositions of the chymotryptic peptides (T1

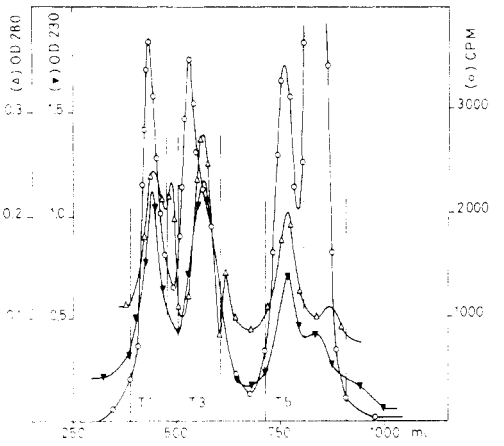

FIGURE 4: Separation of the tryptic peptides of light chain 120 on a (2.5 × 200 cm) Sephadex G-50 column developed in 1 N NH_4OH.

Ch1 to T1 Ch9) are presented in Table IV. Their partial sequences were determined by the dansyl-Edman degradation and are presented in Figure 5.

The chymotryptic digest of peptide ST2 was submitted to high-voltage electrophoresis, and the five major peptides so obtained were analyzed for composition (Table IV) and N-terminal sequence (Figure 6).

Discussion

The complete variable-region sequence of an anti-*Micrococcus lysodeikticus* antibody light chain is presented for the first time. Large fragments of the constant region have been sequenced and composition data on the missing parts obtained, allowing a complete sequence of L 120 to be proposed (Figure 7).

The strategy followed to determine the sequence has been the use of trypsin either on the succinylated or on the unmodified light chain. Succinylation of the lysines restricted the number of tryptic peptides, which were ordered on the basis of the known N-terminal sequence of L 120 and the known constant-region sequence of rabbit light chain of the b4 allotype and κ_B subtype. Light chain 120 contains only three arginine residues, one of which is located at position 208 in the constant regions of all light chains of allotype b4 (Appella et al., 1973; Chen et al., 1974). Of the two remaining arginine residues, one is most probably located at position 61, as has been the case for all rabbit κ chains regardless of their allotype (Margolies et al., 1975). On considering the sizes of the fragments obtained by trypsinolysis of the succinylated light chain, the small 11-residue peptide ST3 is easily placed in the variable region between the N-terminal peptide (1–50) ST2 and the large fragment ST1, which starts at position 62 and comprises the constant region up to position 211.

Fourteen complete rabbit antibody light-chain variable regions are now available for comparison. These are lined up in Figure 7. As indicated earlier (Margolies et al., 1975), the correlation between the hypervariable region sequence and antigen-binding specificity does not appear to be obvious from a comparison of chains of the apparent same specificity, in contrast to the striking similarities observed between anti-phosphorylcholine antibodies or anti-arsonate hapten antibodies and myeloma proteins from inbred strains of mice. Part of the explanation for the difference between the two systems may reside in variability due to the genetic diversity of the rabbits. This interpretation is supported by recent data (Braun et al., 1976) on anti-streptococcal antibodies raised in rabbits from partially inbred families. Although sequences were only

TABLE IV: Amino Acid Composition of Peptides Produced by Tryptic (T), Chymotryptic (CH), and Acid Hydrolysis (A) Produced Peptides.[a]

amino acid	T3.1[b]	T3.2[b]	T3.4[b]	T3.8[b]	T5.1[b]	T5.2[b]	T5.3[b]	ST2-Ch1[b]	ST2-Ch2[b]	ST2-Ch3[b]	ST2-Ch4[b]	ST2-Ch5[b]
CMCys	0.8 (1)		0.8 (1)				1.2 (1)	0.8 (1)				
Asp	3.3 (3)	1.1	4.4 (4)	3.8 (4)	0.2	1.1 (1)	0.3	0.4	0.2	0.2		0.4
Thr	3.8 (4)	3.7 (4)	5.6 (6)	5.6 (6)	1.0 (1)	2.6 (3)	1.0 (1)	5.0 (5)		1.0 (1)		
Ser	0.3	1.8 (2)	4.5 (5)	1.1 (1)	4.0 (4)	2.0 (2)		4.6 (5)		0.2	0.3	
Glu	1.1 (1)	2.9 (3)	2.2 (2)	3.3 (3)	0.8	1.4 (1)	1.0 (1)	4.4 (5)	3.0 (3)	3.2 (3)	3.0 (3)	3.0 (3)
Pro	3.6 (4)	1.1 (1)	0.9 (1)	0.9 (1)		1.6 (1)		1.2 (1)	2.2 (2)	2.9 (3)	2.7 (3)	2.6 (3)
Gly	2.2 (2)	2.2 (2)		3.3 (2)	1.0 (1)	1.2 (3)	0.2	3.0 (3)	2.0 (1)	2.2 (2)	1.2 (1)	2.0 (1)
Ala	4.5 (5)	2.7 (3)	0.8 (1)		1.4 (2)			2.3 (2)		1.2 (1)	0.3	1.1 (1)
Val	5.6 (6)	2.7 (3)		2.9 (3)	1.0 (1)			3.0 (3)	0.4			
Ile	1.8 (2)	0.8 (1)		0.9 (1)				1.4 (2)	1.0 (1)	0.6 (1)	0.7 (1)	
Leu	1.1 (1)	1.1 (1)	2.9 (3)		0.7 (1)			2.1 (2)	1.7 (2)	2.9 (3)	2.0 (2)	1.0 (1)
Tyr	0.2		1.8 (2)	0.8 (1)			1.0 (1)	1.0 (1)	1.2 (2)	2.8 (3)	0.7 (1)	0.7 (1)
Phe	(1)	0.9 (1)		0.9 (1)		0.6 (1)						
His			0.8 (1)									
Lys	0.9 (1)	1 (1)	1 (1)	1 (1)			1.0 (1)	1.0 (1)	1.7 (2)	2.0 (2)	1.8 (2)	1.7 (2)
Arg	1.1				0.9 (1)	1.0 (1)			1.0 (1)	2.3 (1)	1.0 (1)	
Trp[b]	0.1									1[b] (1)		1[b] (1)
total	30.3 (31)	22.0 (22)	25.7 (27)	24.5 (24)	11.0 (11)	12.6 (14)	5.7 (5)	30.2 (31)	14.4 (15)	21.5 (21)	13.7 (14)	12.9 (13)

	T1[b]	T1-Ch1[b]	T1-Ch5[b]	T1-Ch7[b]	T1-Ch3[b]	T1-Ch6[b]	A2	T1-Ch9[b]
CMCys	2.1 (2)		0.9 (1)	1.0 (1)			2.0 (2)	
Asp	0.9 (1)	0.6	1.2 (1)				2.2 (2)	
Thr	5.9 (6)	1.1 (1)	3.1 (3)	0.9 (1)		(1)	6.2 (6)	1.0 (1)
Ser	4.9 (5)		1.0 (1)		2.0 (2)	(2)	5.0 (5)	
Glu	5.0 (5)	0.8 (1)	1.0 (1)	1.0 (1)	0.8 (1)	(2)	5.6 (5)	1.1 (1)
Pro								
Gly	7.7 (8)	3.0 (3)	1.0 (1)	1.0 (1)		(3)	7.8 (9)	3.3 (3)
Ala	4.4 (4)	1.8 (3)	2.9 (3)		1.1 (1)	(1)	4.4 (4)	
Val	4.3 (4)	0.6	1.2 (1)			(3)	4.4 (4)	2.8 (3)
Ile	1.1 (1)		0.9 (1)				1.0 (1)	
Leu	1.4 (1)		1.1 (1)				1.0 (1)	
Tyr	3.8 (4)		1.0 (1)	3.0 (3)		(1)	3.6 (4)	
Phe	2.0 (2)				0.7 (1)	(1)	2.6 (3)	
His								
Lys	1.0 (1)	1.0 (1)				(1)	2.0 (2)	1.0 (1)
Arg								
total	44.5 (44)	8.9 (9)	15.3 (15)	7.0 (7)	4.6 (5)	(15)	47.8 (48)	9.2 (9)

[a] Values are in moles/mole of peptide. [b] As determined by sequence is in parentheses.

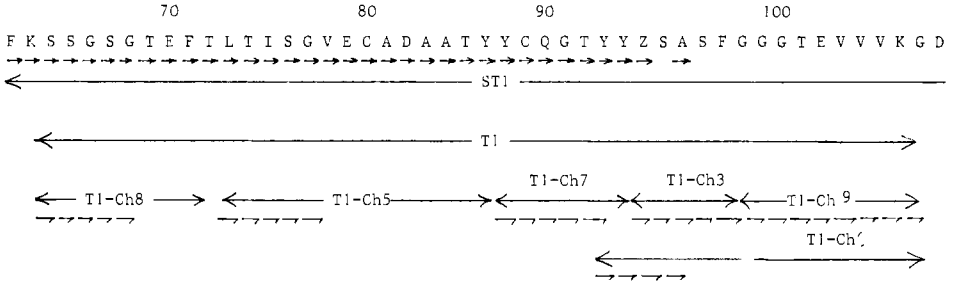

FIGURE 5: Sequence results obtained by dansyl-Edman degradation of peptides derived from peptide ST1 by digestion with chymotrypsin and subsequent separation by high-voltage electrophoresis on paper. The sequence of ST1 between positions 62 and 96 was also determined with a sequenator.

completed for a few chains, the results seem to imply a more limited variability than that observed for rabbits of a different origin.

The comparison of the complete light chains allows the following conclusions: (1) rabbit light chains display only two hypervariable regions, between residues 29 and 32 and between residues 92 and 97. Positions 50 to 56, known to mark the second hypervariable region in human κ chains, are not hypervariable in rabbit light chains. Crystallographic studies have indicated that this region does not necessarily participate in the antigen binding sites (Poljak et al., 1974; Schiffer et al., 1971; Segal et al., 1974). (2) A limited variability of the first five positions of the N-terminal region is observed but cannot be correlated with amino acid substitutions in the remainder of the variable region. (3) Although rabbit κ light chain subgroups have been defined (Cannon et al., 1976), they are not selected preferentially in relation to antibody specificity, nor can they be correlated with the length of the hypervariable

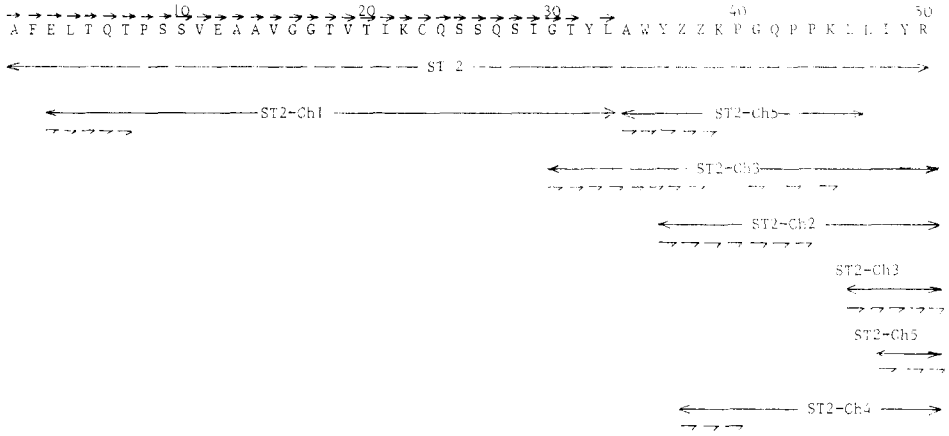

FIGURE 6: Sequence results obtained by dansyl-Edman degradation of peptides derived from peptide ST2 by digestion with a chymotrypsin and subsequent separation by high-voltage electrophoresis on paper. The sequence of ST2 between positions 1 and 33 was also determined by automated degradation in a sequenator.

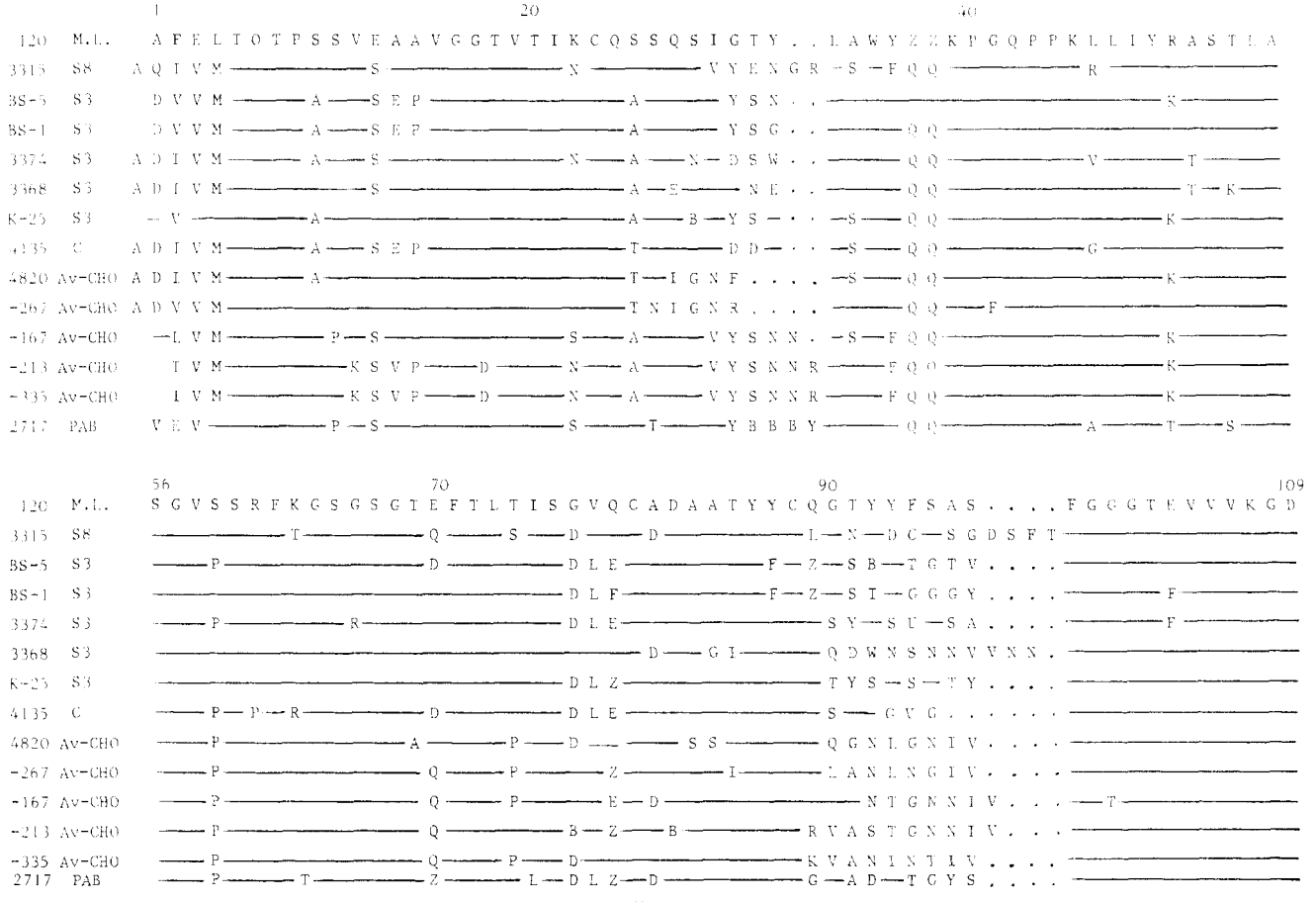

FIGURE 7: Comparison of the complete variable-region sequences of antibody light chains of various specificities. Data are from Margolies et al. (1975) for chains 3315, 3374, 3368; Jaton (1974, 1975) for chains BS-5, BS-1, and K-25; Chen et al. (1974) for chain 4135; Braun et al. (1976) for chains 4820, 267, 167, 213 and 335; and Appella et al. (1973) for chain 2717. Antibody specificities are: M.L., *Micrococcus lysodeikticus*; S3 or S8, pneumococcus type 3 or type 8; C, streptococcus C; Av-CHO, streptococcus A variant; PAB, *p*-azobenzoate.

regions.

Three complete constant regions of κ light chains of allotype b4 are now available for comparison (Chen et al., 1974; Margolies et al., 1975). All three appear to be identical, with the possible exception of certain side-chain amide group assignments. This identity is remarkable in view of the fact that several substitutions have been reported for three positions in the chain. At position 173, Strosberg et al. (1972) reported a Val or a Leu residue, a result previously obtained by Frangione (1969). In the three light chains found to be identical here, this position is occupied by an Asn residue. More recently, Sogn & Kindt (1976) described the inherited appearance of a Ser-Ala-Asp-Leu sequence between positions 121 and 124, instead of the more common Ala-Ala-Asp-Gln sequence substitutions. These substitutions may correspond to the serological subtypes reported previously by Van der Loo et al. (1975).

The general structure of the rabbit light chain, as defined by the three complete chains now available, is compatible with the three-dimensional structure proposed for human myeloma light chains (Poljak et al., 1974; Schiffer et al., 1971; Segal et

al., 1974). The disulfide bond linking residues 80 to 170 (Strosberg et al., 1975), which is absent in human chains, corresponds to residues which are specially adjacent, according to the crystallographic results. Residues such as Gly or Pro, known to be involved in β turns of the polypeptide chain, are found in rabbit chains at the same positions as in human or murine chains. Several of these residues are parts of stretches which are highly conserved in all mammalian light chains.

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