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Light Chain Variable Region Sequence of Rabbit Antipneumococcal Type III Polysaccharide Antibody 3368†

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ABSTRACT: The amino acid sequence of the amino-terminal 111 residues (variable region) for the light chain of the homogeneous rabbit antipneumococcal type III polysaccharide antibody 3368 was determined. This sequence was obtained principally through automated Edman degradations of the intact light chain and of peptides generated by tryptic digestion of the citraconylated light chain. With these methods only 2 μmol of purified light chain was required to determine the reported sequence. When compared with the light chains of four other antipneumococcal type III polysaccharide antibodies, the 3368 light chain exhibits a unique sequence in those segments of the variable region that contribute to formation of the antigen binding site (complementarity-determining regions) (10 or 11 residue differences in 12 positions). The 3368 light chain also demonstrates an insertion of three residues relative to the other four light chains in the complementarity-determining region at positions 89 to 98. These five light chains have greater than 80% sequence homology for the portion of the variable region which is not involved in antigen binding (framework).

The considerable interest in the variable domain structures of immunoglobulin (Ig)[1] molecules stems in large measure from the demonstration that the antigen binding site is contained within the variable domain. High resolution x-ray crystallographic analyses have demonstrated that the variable regions of Ig light and heavy chains fold together to form a compact domain, and that antigen is specifically bound within a well-defined site formed by the interaction of the two variable regions (Poljak et al., 1974; Segal et al., 1974). At least two discrete polypeptide segments of the light chain and three discrete segments from the heavy chain variable region form the walls of the antigen binding site. These complementarity-determining regions of the Ig variable domain correspond to the hypervariable regions identified by statistical analyses of variable region amino acid sequences from human and murine Igs (Wu & Kabat, 1970; Kabat & Wu, 1971; Capra & Kehoe, 1974) and, more recently, from rabbit antibody light chains (Margolies et al., 1975). The amino acid sequence diversity among these complementarity-determining regions presumably accounts for the capacity of the immune system to respond to a seemingly unlimited variety of antigenic stimuli with the production of specific antibodies, and it is also re-

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[1] Abbreviations used: Ig, immunoglobulin; Pth, phenylthiohydantoin; CM, carboxymethyl; Gdn·HCl, guanidine hydrochloride.

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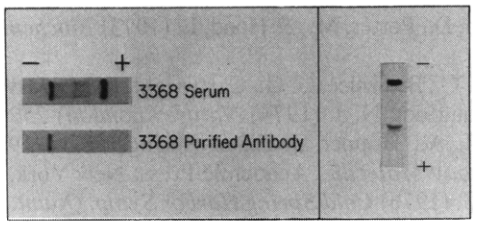

FIGURE 1: (Left panel) Cellulose acetate electrophoresis of hyperimmune antiserum from rabbit 3368 and of DE-52 cellulose purified antibody. (Right panel) Alkaline urea polyacrylamide disc gel electrophoresis of fully reduced and alkylated 3368 antibody. Approximately 100 μg was applied to the gel.

sponsible for the defined specificity characteristic of individual antibody molecules (Landsteiner, 1946). Comparative analyses of variable region amino acid sequences for antibodies elicited by the same antigen should enable a definitive assessment of the structural correlates of antibody specificity and diversity.

In the present report we describe the amino acid sequence obtained for the light chain variable region (amino-terminal 111 residues) of a monoclonal antibody elicited in rabbit 3368 by repetitive immunization with the pneumococcal type III vaccine. The purified antibody is specific for the type III capsular polysaccharide, a linearly repeating polymer of cellobiuronic acid. The 3368 light chain is type κ, subtype B, and carries the b4 allotype. The strategy for sequence determination depended on lengthy sequenator degradations and on tryptic cleavage of the light chain at arginine residues after citraconylation of lysine residues. Using these methods, only 2 μmol of light chain was required for determining the reported sequence. The 3368 variable region sequence is compared with those of other rabbit light chains of identical isotype and allotype and also obtained from antibodies elicited by the type III pneumococcal vaccine.

Experimental Section

Materials

Iodoacetic acid (Eastman Kodak) was recrystallized four times from chloroform before use. Iodoacetamide (Sigma Chemical Co.) was recrystallized twice from water. Dithiothreitol (Sigma Chemical Co.), [^{3}H]iodoacetic acid (206.6 mCi/mM; New England Nuclear), and [^{14}C]iodoacetamide (13.8 mCi/mM; New England Nuclear) were used without additional purification. Phenylmethanesulfonyl fluoride was purchased from Sigma Chemical Co. Citraconic anhydride was obtained from Aldrich Chemical Co. and was periodically redistilled in vacuo. Trypsin (α-1-tosylamido-2-phenylethyl chloromethyl ketone treated) and carboxypeptidase B (diisopropyl fluorophosphate treated) were products of Worthington Biochemical Corp. Gel filtration media were purchased from Pharmacia Fine Chemicals, and DE-52 cellulose was obtained from Whatman. All fluorescamine (Roche Diagnostics) solutions were prepared in Sequenal grade acetone (Pierce Chemical Co.). Gdn·HCl was Schwarz/Mann enzyme grade. Sequenator reagents, thin-layer chromatographic supports, and gas-liquid chromatographic supports were previously described (Brauer et al., 1975).

Methods

General. The preparation of the type III pneumococcal vaccine, the immunization of rabbit 3368 with type III vaccine, and the screening of antisera for antibody components of monoclonal origin by cellulose acetate electrophoresis were as previously described (Chen et al., 1973). Quantitative precipitin analysis was done according to Kabat & Mayer (1961). Alkaline urea–polyacrylamide discontinuous gel electrophoresis of completely reduced and alkylated antibody was performed as described by Reisfeld & Small (1966). The *b*-locus allotype of the 3368 serum Ig and purified antibody was determined by radioimmunoassay using *N*-ethylchloroformate cross-linked allotypic antisera (Tosi & Landucci-Tosi, 1973) and by immunodiffusion (Ouchterlony, 1953). Allotype specific antisera were the generous gifts of Dr. Charles Todd.

The fluorescamine assay was modified from Udenfriend et al. (1972) as follows: 10–50 μL aliquots of a sample solution were added to 0.3 mL of 0.05 M sodium borate (pH 8.2) buffer, followed by addition of 0.2 mL of a 0.03% solution of fluorescamine in acetone with vigorous mixing. Fluorescence was measured with an Aminco Bowman spectrophotofluorometer (American Instrument Co., Inc.). The excitation wave-length was 390 nm and emitted fluorescence was measured at 475 nm.

Purification of the 3368 Antibody. Antiserum (10 mL) from rabbit 3368 that exhibited a major discrete component in the γ globulin region on cellulose acetate electrophoresis (see Figure 1) was dialyzed against 6 L of 1 mM potassium phosphate (pH 7.5) at 4 °C for 72 h with two changes of dialyzate every 24 h. The serum was then centrifuged at 12 000*g* for 30 min and the supernatant applied to a column (2.6 × 40 cm) containing DE-52 cellulose equilibrated with 1 mM potassium phosphate (pH 7.5). The column was eluted with a 1 column volume of the 1 mM starting buffer and then with a linear gradient (total gradient equal to 3 column volumes) to 10 mM potassium phosphate (pH 7.5). This gradient was immediately followed by a second gradient to 0.1 M potassium phosphate (pH 7.5). The column was then elute with a 1 column volume of 0.5 M potassium phosphate (pH 7.5).

Separation of Light and Heavy Chains. A 2–3% solution of antibody in 0.5 M Tris-HCl (pH 8.2) buffer was reduced by the addition of dithiothreitol to a concentration of 20 mM. The reaction mixture was stirred under N_2 for 2 h, at which time iodoacetic acid containing 1 mCi of [^{3}H]iodoacetic acid was added to a concentration of 50 mM. The pH stat (Radiometer Autoburette, ABU 11) was employed for addition of 1 N NaOH to maintain a constant pH 8.2. After 30 min the reaction was stopped by acidification to pH 3 with 6 N HCl. The mildly reduced and alkylated antibody was immediately dialyzed against 1 M acetic acid for 4–8 h and then dialyzed overnight against 0.1 M sodium acetate–5 M Gdn·HCl (pH 5.5). The light and heavy chains were separated by Sephadex G-100 gel filtration in 0.1 M sodium acetate–5 M Gdn·HCl (pH 5.5). The isolated polypeptide chains were dialyzed extensively against water and lyophilized.

Complete reduction and alkylation of light chains employed reaction conditions identical with those described above except that 0.5 M Tris-HCl–7 M Gdn·HCl (pH 8.2) was the buffer and iodoacetamide containing 50 μCi of [^{14}C]iodoacetamide was the alkylating reagent.

Citraconylation of Completely Reduced and Alkylated Light Chain. A 2% solution of completely reduced and alkylated light chain in 0.1 M sodium borate–7 M Gdn·HCl (pH 9.0)[2] was reacted with an aliquot of citraconic anhydride corresponding to a tenfold molar excess relative to light chain

[2] Upon standing overnight, the 0.1 M sodium borate, 7 M Gdn·HCl (pH 9.0) buffer forms a powderlike precipitate that can be removed by centrifugation.

lysine content. The pH stat was employed for the addition of 5 N NaOH to maintain a constant pH of 9.0. Following pH stabilization, a 5 μL aliquot of the reaction mixture was assayed for the presence of primary amines by reaction with fluorescamine.[3] Additional aliquots of citraconic anhydride were added until a blank value was attained in the fluorescamine assay. This required a 30-fold molar excess of citraconic anhydride to lysine content.

Following complete citraconylation, the sample was dialyzed overnight at 4 °C against 600 volumes of 1% ammonium bicarbonate (pH 9.0) to remove excess citraconic acid and nonvolatile salts and then for 4–6 h at room temperature against 600 volumes of 1% ammonium bicarbonate (pH 8.5) in preparation for tryptic digestion.

Tryptic Peptides from the Citraconylated Light Chain. Prior to digestion with trypsin, a 25-μL aliquot of a 1% solution of citraconylated light chain in 1% ammonium bicarbonate (pH 8.5) was assayed for free amino groups by reaction with fluorescamine. Free amino groups were not detected and therefore, tryptic digestion at 25 °C was begun with the addition of a 1:200 enzyme:light chain (w/w) aliquot of trypsin. At 2 and 3 h after the initiation of digestion, additional 1:200 enzyme:light chain (w/w) aliquots of trypsin were added. Trypsin digestion was terminated 30 min after addition of the last aliquot of trypsin by adding phenylmethanesulfonyl fluoride (400-fold molar excess relative to trypsin). Thirty minutes after addition of the phenylmethanesulfonyl fluoride, the mixture was lyophilized. The lyophilized digest was dissolved in a minimum volume of 10% acetic acid–7 M Gdn·HCl and incubated overnight at 4 °C to remove citraconyl groups. Peptides were fractionated by Sephadex G-75 gel filtration in 0.1 M sodium acetate–5 M Gdn·HCl (pH 5.5). With the exception of the largest peptide, all peptides obtained from the tryptic digest of the citraconylated light chain were desalted by Sephadex G-10 gel filtration on a 3.5 × 100 cm column in 0.01 N NH_4OH. The largest peptide was desalted[4] by first adjusting the peptide solution to pH 3.0 with glacial acetic acid and then dialyzing against 1 M acetic acid at 4 °C.

Following removal of citraconyl groups certain peptides were further digested with trypsin. To a solution of peptide (0.1 to 0.5 mM) in 1% ammonium bicarbonate (pH 8.0) at 37 °C three 1:200 enzyme:peptide (M/M) aliquots of trypsin were added at 0, 2, and 3 h. The digest was lyophilized 30 min after the addition of the last aliquot of trypsin.

Carboxypeptidase B Digestion. For each peptide, two samples (20–30 nmol) were each dissolved in 0.3 mL of 1% ammonium bicarbonate (pH 8.2). To each sample was added 5 μg of carboxypeptidase B (1:200 molar ratio of enzyme to peptide), as determined spectrophotometrically from a freshly prepared solution of the enzyme (Ambler, 1972). Immediately following addition of the enzyme, one of the samples was acidified by addition of 0.5 mL of 1 M acetic acid and lyophilized. The other sample was incubated at 37 °C for 30 min and then acidified with 0.5 mL of 0.1 M acetic acid and lyophilized. Free amino acids were determined directly on the amino acid analyzer. A control, containing enzyme but no peptide, was included in each experiment for determining background amino acids.

Amino Acid Analyses. Amino acid compositions were determined with a Beckman 120 B analyzer equipped with a high sensitivity cuvette and an Infotronics digital integrator. Samples in constant boiling HCl and containing 5 μL of butanedithiol were sealed in vacuo and hydrolyzed at 110 °C for 24 h.

Amino Acid Sequence Determination. Automated Edman degradations were performed in a modified Beckman 890 B sequencer and employed either a 1 M Quadrol program or a 0.1 M Quadrol protein microsequence program (Brauer et al., 1975). Peptides of fewer than 11 residues were degraded by manual Edman degradation as modified by Niall & Potts (1970).

With the exception of arginine, all amino acids identified in the 3368 light chain were identified as their phenylthiohydantoin (Pth) derivatives. Conversion of thiazolinone amino acid derivatives to their Pth derivatives, extraction of Pth-amino acids with ethyl acetate, and the quantitative identification of Pth-amino acids by gas–liquid chromatography have been described (Brauer et al., 1975).

Gas–liquid chromatographic identifications were supplemented by thin-layer chromatography on silica gel plates according to Edman (1970) or on polyamide sheets (Summers et al., 1973). For selected cycles, gas–liquid chromatographic identifications were confirmed by hydrolysis of Pth-amino acids with HI followed by identification on the amino acid analyzer (Smithies et al., 1971). This method was employed primarily to confirm the distinction between Pth-leucine and Pth-isoleucine obtained by gas–liquid chromatographic analysis of silylated derivatives. For all residues reported, there was agreement between primary identification by gas-liquid chromatography and identification by secondary methods.

In manual degradations of small peptides, arginine at the carboxy terminus was identified directly on a Durrum D 500 high sensitivity amino acid analyzer following cleavage of the penultimate residue.

Peptide Nomenclature and Residue Numbering. Peptides generated by tryptic digestion after citraconylation of the completely reduced and alkylated 3368 light chain are designated by the letters CT followed by a number denoting the linear order of that peptide from amino to carboxy terminus of the light chain (e.g., CT1, CT2, etc.). Fragments of these peptides produced by trypsin digestion after removal of citraconyl groups are designated by a hyphen followed by the letter T and a numeral denoting linear alignment in the parent peptide, following designation of the parent peptide (e.g., CT1-T1). Amino acid residue position number is based on homology with the human κ chain prototype sequence Ag (Putnam et al., 1966).

Results

Antibody Purification. The fractionation of antiserum (10 mL) from rabbit 3368 by DE-52 cellulose chromatography yielded an Ig fraction that eluted within the 1 mM to 10 mM potassium phosphate gradient. Cellulose acetate electrophoresis of this fraction indicated a single electrophoretic species (Figure 1) representing the major IgG component present in the 3368 antiserum. The amount of IgG in this fraction was estimated to be 240 mg by absorbance at 280 nm ($A_{280}^{1\%}$ = 14 was assumed), a quantity that agrees with a serum antibody concentration of 28 mg/mL as determined by quantitative precipitin analysis. The antibody activity of the purified fraction was confirmed by quantitative precipitation analyses

[3] Experiments comparing the fluorescamine assay with the dinitrofluorobenzene assay (Schroeder & Legette, 1953; Freedlender & Haber, 1972) validated the reliability of the fluorescamine method for assaying the modification of primary amines.

[4] In earlier experiments we found that trypsin and the largest peptide obtained by tryptic digestion of the citraconylated light chain coelute on G-75 gel filtration. If this peptide is desalted at neutral or alkaline pH, the trypsin is renatured and cleavage at lysyl peptide bonds may occur. Terminating the tryptic digestion of the citraconylated light chain by adding phenylmethanesulfonyl fluoride and desalting the largest peptide at acidic pH were found to circumvent this problem.

TABLE I: Automated Edman Degradation 3368 Light Chain.[a]

cycle	Quadrol program 1 M	0.1 M[b]	cycle	1 M	0.1 M
1	A (483)	A (111)	27	S (28)	S (10)
2	D (NQ)[c]		28	E (NQ)	E (NQ)
3	I (489)	I (82)	29	S (33)	S (16)
4	V (354)	V (89)	30	I (65)	I (18)
5	M (378)		31	G (57)	G (12)
6	T (191)		32	N (45)	N (6)
7	Q (NQ)	Q (67)	33	E (NQ)	E (NQ)
8	T (150)		34	L (53)	L (17)
9	P (216)		35	A (48)	A (17)
10	S (156)	S (34)	36	W (17)	W (5)
11	S (143)		37	Y (13)	Y (4)
12	V (190)	V (54)	38	Q (25)	Q (4)
13	S (114)	S (35)	39	Q (29)	
14	A (226)	A (63)	40	K (5)	K (3)
15	A (219)		41	P (18)	P (13)
16	V (223)	V (48)	42	G (17)	G (9)
17	G (187)	G (14)	43	Q (15)	Q (2)
18	G (152)	G (34)	44	---	P (7)
19	T (76)	T (18)	45	P (9)	---
20	V (156)	V (39)	46	K (NQ)	K (2)
21	T (88)		47	L (12)	L (4)
22	I (113)	I (41)	48	L (12)	L (4)
23	K (34)	K (NQ)	49	L/I (16)	L/I (9)
24	---[d]	---	50	---	Y (1)
25	Q (63)	Q (15)	51	---	---
26	A (119)	A (25)	52	A (6)	A (4)

[a] A total of 550 nmol of light chain was used for the 1 M Quadrol degradation; 140 nmol was applied for the 0.1 M Quadrol degradation. Pth-amino acids are represented in a single letter code (IUPAC-IUB Commission on Biochemical Nomenclature, 1968). Yields of Pth-amino acids in nmol are given in parentheses. Quantitation was based on gas-liquid chromatography as previously described (Brauer et al., 1975). All identifications reported were confirmed by thin-layer chromatography and/or amino acid analysis following hydrolysis. [b] Certain cycles from the repeat degradation using 0.1 M Quadrol were not analyzed and are indicated by blanks. [c] Not quantitated. [d] A dashed line indicates that a new Pth-amino acid at that cycle was not found. The failure to identify a Pth-amino acid at cycle 24 is due to the preesence of a half-cystine which is disulfide bonded to a half-cystine at position 88 in the mildly reduced and alkylated light chain.

using the pneumococcal type III polysaccharide. The pneumococcal type VIII polysaccharide failed to precipitate either the purified DE-52 fraction or the 3368 antiserum.[5]

The antibody purified by DE-52 cellulose chromatography was dialyzed against water and lyophilized. A small amount ($\simeq$2 mg) was then completely reduced and alkylated and subjected to alkaline urea–discontinuous polyacrylamide gel electrophoresis. As shown in Figure 1, the antibody fraction contained a single light chain.

Allotype determinations for both the preimmunization and postimmunization sera revealed that the 3368 rabbit was homozygous for the *b*4 allele at the Ig light chain *b* locus. Likewise, the purified antibody fraction from DE-52 cellulose chromatography of 3368 serum was determined to carry the *b*4 allotype.

Sequencer Degradation of Intact 3368 Light Chain. Following the demonstration of a single light chain by gel electrophoresis (Figure 1), the purified 3368 antibody was reduced (in the absence of denaturants) and alkylated, and light and heavy chains were separated. A total of 550 nmol of light chain was then subjected to sequencer degradation employing a 1 M Quadrol program. At the initial cycle of degradation, 483 nmol of Pth-alanine was obtained representing an 88% amino terminal yield (Table I). Except for cycles 24 and 44, a single Pth-amino acid was identified for 48 cycles of degradations. By homology with other rabbit light chains (Appella et al., 1973; Chen et al., 1974; Jaton, 1974a,b, 1975), the amino acid at cycle 24 is expected to be a half-cystine that is disulfide bonded to a half-cystine at position 88 in the mildly reduced and alkylated light chain. Thus Edman degradation would not be expected to yield a free Pth-amino acid at this cycle. CM-Cys was identified at this position in a peptide isolated from the fully reduced and alkylated light chain (vide infra). The average repetitive yield for this degradation, as quantitated by gas–liquid chromatography, was 95%. Subsequently 150 nmol of mildly reduced and alkylated 3368 light chain was subjected to automated Edman degradation using a 0.1 M Quadrol protein program (Brauer et al., 1975). In this experiment the amino-terminal yield was 74% and the average repetitive yield was 96% (Table I). Confirmation of previously identified residues was obtained in this degradation and, in addition, Pth-tyrosine and Pth-alanine were identified at cycles 50 and 52, respectively.

Purification of Tryptic Peptides from the Citraconylated Light Chain. Following the demonstration of a single sequence for the amino-terminal 52 residues, 1 μmol of the 3368 light chain was completely reduced and alkylated, citraconylated, digested with trypsin, and the peptides were fractionated by Sephadex G-75 gel filtration; the results are shown in Figure 2. Four fractions were resolved, consistent with amino acid analysis of the intact light chain that indicated the presence of three arginine residues (Table II). Fractions A, B, C, and D were each pooled, as indicated in Figure 2, and desalted. As shown in Table II, the amino acid compositions of the G-75 fractions account for the composition of the intact light chain.

[5] Functional characterization of the 3368 antibody and other antibodies currently being studied in our laboratory will be reported by the authors and M. Cronlund.

TABLE II: Amino Acid Composition of 3368 Light Chain and Tryptic Peptides from the Citraconylated Chain.[a]

amino acid	fraction[b] A (CT3)	B (CT1)	C (CT2)	D (CT4)	intact light chain
Lys	6.1 (6)	2.9 (3)	1.0 (1)		10.0 (10)
His	1.0 (1)				1.0 (1)
Arg	1.0 (1)	0.9 (1)	1.1 (1)		3.0 (3)
CM-Cys	5.0 (5)	1.0 (1)		0.6 (1)	7.2 (7)
Asp	18.9 (19)	2.3 (2)		1.2 (1)	21.8 (22)
Thr	23.6 (24)	4.0 (4)			26.4 (28)
Ser	11.1 (12)	4.8 (5)	3.8 (4)		18.7 (21)
Glu	13.9 (14)	7.2 (7)			21.5 (21)
Pro	5.8 (6)	3.8 (4)			10.0 (10)
Gly	13.1 (13)	4.2 (4)	1.2 (1)	1.2 (1)	18.9 (19)
Ala	7.1 (7)	4.9 (5)	1.9 (2)		14.0 (14)
Val	18.2 (18)	4.0 (4)	1.0 (1)		23.5 (23)
Met		1.2 (1)			1.3 (1)
Ile	4.3 (5)	3.7 (4)			7.8 (9)
Leu	4.8 (5)	2.9 (3)	1.0 (1)		9.3 (9)
Tyr	5.9 (6)	2.1 (2)			8.1 (8)
Phe	6.0 (6)				5.9 (6)
Trp	ND[c] (2)	ND (1)	ND (0)	ND (0)	ND (3)
total	150	51	11	3	215
yield of peptide	77%	83%	76%	45%	

[a] Compositions are calculated from 24-h hydrolyses assuming molecular weights of 15 000 and 5000 for fractions A and B, respectively, 11 residues for fraction C, and 3 residues for fraction D. A molecular weight of 22 500 was assumed for the fully reduced and alkylated light chain. The values shown are not corrected for hydrolytic losses. Residues found in the sequence analysis or assumed from the published constant region sequence of rabbit allotype *b4* light chains (Chen et al., 1974) are given in parentheses. [b] Fractions A–D are identified in Figure 2. Peptide designations (CT1, CT2, CT3, CT4) in parentheses indicated fragment alignment (see text). [c] Tryptophan was not determined.

Alignment of Tryptic Peptides from the Citraconylated Light Chain. The presence of ^3H radioactivity (Figure 2) and the absence of arginine in the amino acid composition of fraction D (Table II) identify this fraction as containing the carboxy-terminal fragment CT4 of the light chain. In the native antibody molecule, the [^3H]CM-Cys in this peptide corresponds to the disulfide linkage between heavy and light chains and is reduced and alkylated in the absence of denaturing reagents (O'Donnell et al., 1970). Manual Edman degradation of this peptide yielded a glycine amino-terminus consistent with the sequence Gly-Asp-Cys reported for the carboxy terminal three residues of rabbit light chains having the *b4* allotype (Chen et al., 1974). This peptide is generated by tryptic cleavage of an arginyl peptide bond at position 211–212 in the constant region of rabbit *b4* light chains (Chen et al., 1974).

Carboxypeptidase B digestion of the peptides in fractions A, B, and C (Figure 2) established arginine as the carboxy-terminal residue for each. Manual Edman degradation (5 cycles) of the peptide in fraction B gave the sequence Ala-Asp-Ile-Val-Met. As this sequence is identical with that obtained for the amino-terminal five residues of the intact light chain (Table I), fraction B is the amino-terminal tryptic fragment CT1 of the citraconylated light chain. The ordering of fraction C as CT2 and fraction A as CT3 is based on the knowledge that the arginine at position 211 is the only arginine in the rabbit *b4* light chain constant region (Chen et al., 1974). Therefore, the fragment that is contiguous with the carboxy-terminal tripeptide CT4 must be a minimum of 100 residues in length. Since only fraction A satisfies this criterion (Figure 2 and Table II), it is the CT3 tryptic fragment in the citraconylated light chain. Fraction C is thus identified as the CT2 fragment. The sequences obtained for fraction C and for the amino-terminal 49 residues of fraction A confirm this ordering (vide infra).

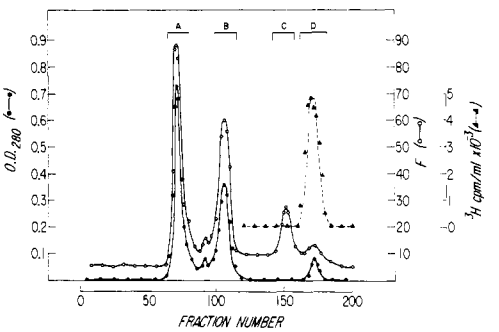

FIGURE 2: Fractionation of tryptic peptides from citraconylated, fully reduced and alkylated 3368 light chain (1 μmol) on Sephadex G-75. Column dimensions were 2.6 × 95 cm, column solvent was 0.1 M sodium acetate–5 M Gdn·HCl (pH 5.5), and fraction volume was 2.7 mL. Column effluent was monitored by optical density at 280 nm, by fluorescamine assay of 10-μL aliquots from each fraction (F in the figure), and by liquid scintillation counting (25-μL aliquots from each fraction) in Bray's solution. In the interest of clarity, ^{14}C counts (which were found only in fractions A and B) are not plotted.

As shown in Table II, the yields of all variable region peptides (CT1, CT2, and CT3) were greater than 75%. The lower yield of CT4 was primarily the result of difficulties during desalting. This fraction eluted as a broad peak in Sephadex G-10 gel filtration, a portion of which was not separated from salts.

Completion of the Sequence of CT1. The amino-terminal fragment CT1 (500 nmol) was digested with trypsin and the digest fractionated by Sephadex G-25 (fine) gel filtration in 0.01 N NH_4OH. Two fractions were resolved as indicated in Figure 3. Based on the sequencer degradation of the intact light chain (Table I) and the composition of CT1 (Table II), three peptides corresponding to cleavage at lysine residues identified at cycles 23 and 46 were expected. The lysine identified at cycle

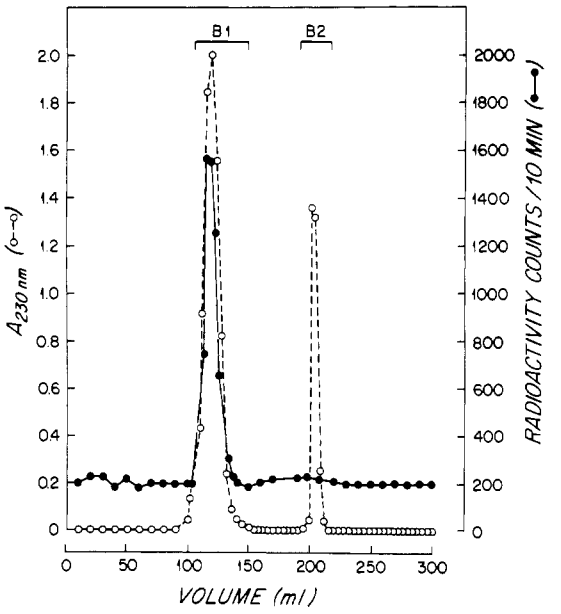

FIGURE 3: Sephadex G-25 (fine) gel filtration of the tryptic digest of CT1 (500 nmol). Column dimensions were 1.2 × 190 cm, column solvent was 0.01 N NH_4OH, and 1-mL fractions were collected. The column effluent was monitored by optical density at 230 nm and by liquid scintillation counting (25-μL aliquots from each fraction) in Bray's solution.

40 is followed by a proline and is not expected to be cleaved by trypsin (Smyth, 1967).

Manual Edman degradation of fraction B1 (Figure 3) yielded two Pth-amino acids at each of four consecutive cycles (Table III). One of these sequences, Ala-Asp-Ile-Val, is identifiable as the amino-terminal four residues of the 3368 light chain (Table I) and corresponds to the CT1-T1 peptide. The other sequence, Cys-Gln-Ala-Ser, is derived from cleavage at the lysine residue identified at cycle 23 in the sequencer degradation of the intact light chain (Table I) and identifies CT1-T2. The identification of [^{14}C]CM-Cys at cycle 1 of fraction B1 established half-cystine as the residue corresponding to cycle 24 of the sequencer degradation of the mildly reduced and alkylated light chain (Table I).

The amino acid composition of fraction B2 (Arg (1.1), Ile (1.0), Leu (1.9), Tyr(1.0)) identifies it as the carboxy-terminal tryptic peptide of CT1, CT1-T3, as it contains the single arginine residue of CT1 (Table II). Manual Edman degradation of the pentapeptide CT1-T3 yielded the sequence Leu-Leu-Ile-Tyr-Arg (Table III), indicating that this peptide arises from tryptic cleavage at the lysine residue identified at cycle 46 of the sequencer degradation of the intact light chain (Table I and Figure 5). The identification of Pth-isoleucine at cycle 3 and of arginine as the carboxy-terminal residue of this peptide combined with the data shown in Table I completes the sequence of the amino-terminal 52 residues of the 3368 light chain.

Sequence of CT2. The amino acid analysis of CT2 is shown in Table II. The peptide (350 nmol) was subjected to sequencer degradation with the results shown in Table IV. Because of the poor repetitive yield due to extractive loss of the peptide from the spinning cup, only the amino terminal 8 residues could be identified. Amino acid analysis (Table II) and carboxypeptidase B digestion suggested the remaining sequence to be Ser-Ser-Arg. This was confirmed by digesting the peptide (300 nmol) with trypsin and subjecting the mixture to manual Edman degradation (Table IV). Both Pth-alanine and Pth-leucine were identified at cycle 1. For cycles 2 through 7 only a single Pth-amino acid was identified. Free arginine was

TABLE III: Manual Edman Degradation of Fractions B1 and B2.[a]

cycle	Pth-amino acids[b] B1	B2
1	Ala (32), CMCys (30)	Leu (88)
2	Asp (NQ), Gln (NQ)	Leu (70)
3	Ile (27), Ala (8)	Ile (66)
4	Val (14), Ser (6)	Tyr (23)
5		Arg[c] (9)

[a] Fractions B1 and B2 are products of tryptic digestion of peptide CT1 (see Figure 5). [b] Numbers in parentheses are amounts (nmol) of the respective Pth-amino acids. Pth-aspartic acid and Pth-glutamine at cycle 2 of fraction B1 were not quantitated (NQ). [c] Free arginine was determined using the amino acid analyzer following cleavage at cycle 4.

TABLE IV: Edman Degradation[a] of Fraction C (Fragment CT2) from the Tryptic Digest of Citraconylated 3368 Light Chain.

cycle	Pth-amino acids[b] intact CT2	tryptic digest of CT2
1	Ala (312)	Leu (230), Ala (246)
2	Ser (156)	Ala (236)
3	Lys (235)	Ser (37)
4	Leu (182)	Gly (107)
5	Ala (130)	Val (108)
6	Ser (36)	Ser (43)
7	Gly (22)	Ser (51)
8	Val (36)	Arg[c] (10)

[a] The sequence of CT2 was determined in the sequencer using a 0.1 M Quadrol peptide program (Brauer et al., 1975). The sequence of trypsin digested CT2 was determined by manual Edman degradation. [b] Numbers in parentheses are amounts (nmol) of the respective Pth-amino acids. [c] Free arginine was determined on the amino acid analyzer following cleavage at cycle 7.

identified on the analyzer following cleavage at cycle 7. Inspection of Table IV reveals that the sequence Leu-Ala-Ser-Gly-Val-Ser-Ser-Arg, obtained following tryptic digestion of CT2, arises from cleavage of the lysyl-leucyl peptide bond identified at cycles 3 and 4 in the intact CT2. The two degradations complete the sequence of CT2.

The Pth-alanine identified as the amino-terminal residue of CT2 corresponds to the Pth-alanine identified at cycle 52 in the sequenator degradation of the intact light chain (Table I and Figure 5). This confirms the assignment of fraction C from the Sephadex G-75 fractionation (Figure 2) as the CT2 peptide.

Sequencer Degradation of CT3. Following desalting, 350 nmol of CT3 (fraction A, Figure 2) was subjected to sequencer degradation using the 1 M Quadrol program with the results shown in Figure 4. A single Pth-amino acid was identified by both gas–liquid chromatography and thin-layer chromatography on silica gel plates for 49 consecutive cycles. The amino-terminal yield for this degradation was 57% and the repetitive yield was 95%.

The Pth-[^{14}C]CM-Cys identified at cycle 19 and at cycle 27 in the sequencer degradation of CT3 correspond to half-cystines in the intact light chain that are disulfide bonded to half-cystines at position 171 and at position 23, respectively (Strosberg et al., 1975; Jaton, 1974a). The sequence obtained at cycles 41 through 49 begins that portion of rabbit *b4* light chains that has proved invariant (Margolies et al., 1975; Chen et al., 1974; Appella et al., 1973; Jaton, 1974a,b, 1975) with a single exception (Braun et al., 1976). A second sequencer

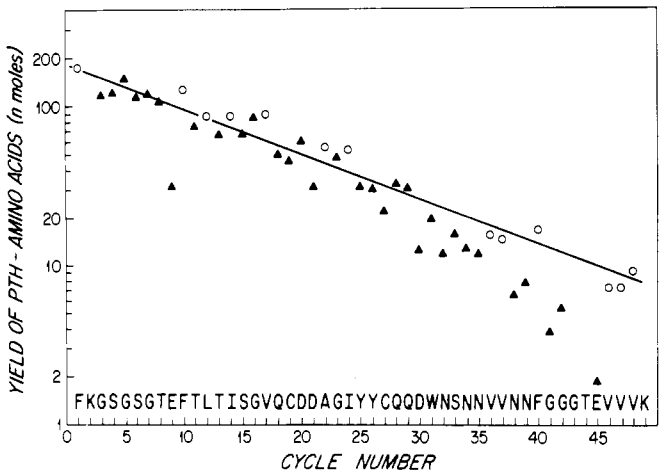

FIGURE 4: Yields of Pth-amino acids as quantitated by gas-liquid chromatography for sequencer degradation of the CT3 fragment (350 nmol). Circles (○) designate stable Pth-amino acid derivatives and triangles (▲) denote unstable Pth-amino acids. The degradation utilized a 1 M Quadrol program.

degradation on CT3 (250 nmol), using the 1 M Quadrol program confirmed every identification reported in Figure 4.

Discussion

The amino acid sequence determined for the amino-terminal 111 residues of the 3368 light chain and location of enzymatic cleavages are shown in Figure 5. As indicated, the determination of the sequence for this portion of the light chain required the use of a single enzyme, trypsin, for the generation of necessary peptides. This afforded a considerable economy of material and labor as compared with classical strategies, and it is due to the ability to obtain lengthy sequenator degradations on intact light chain and large peptides. Of the 111 residues identified in the 3368 light chain, 105 were obtained by automated Edman degradation. In addition to economy in amounts of antibody and the time required to obtain sequence data, extended sequenator degradations reduce the probability of incorrect sequence alignments that are due to inadequate overlap sequences. For example, the 3368 light chain has the sequence Asn-Ser-Asn-Asn-Val-Val-Asn-Asn- at positions 93–97C (Figure 5). The correct assignment of this sequence

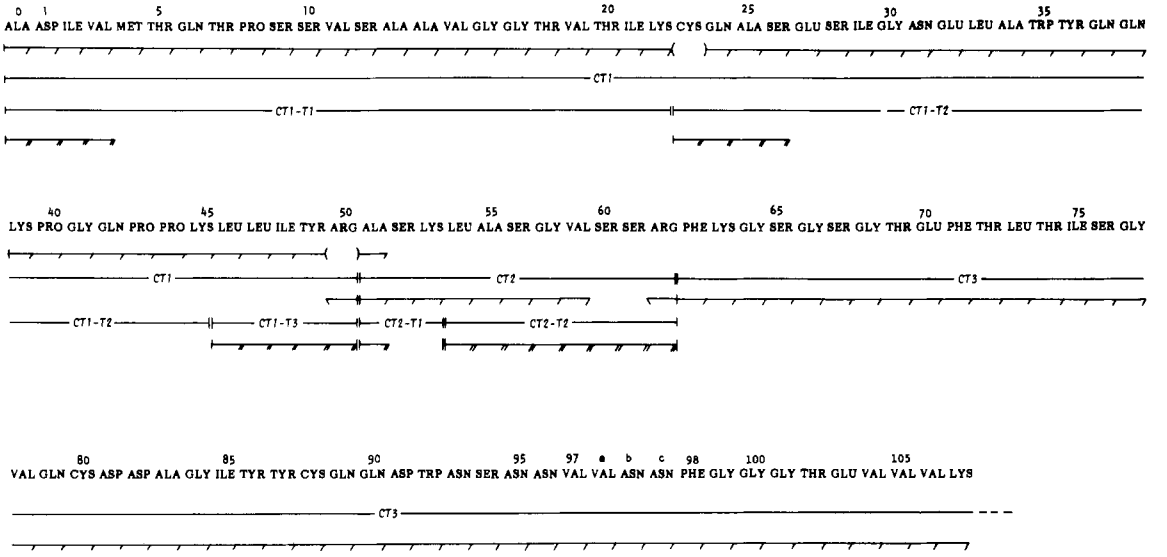

FIGURE 5: Amino acid sequence of the 3368 variable region (positions 0 to 107), alignment of peptides, and locations of enzymatic cleavages. The CT1, CT2, and CT3 fragments were obtained by tryptic digestion of the citraconylated, fully reduced, and alkylated light chain. CT1-T1, -T2, and -T3 were were generated by tryptic cleavage of the CT1 fragment. CT2-T1 and -T2 were produced by tryptic cleavage of the CT2 peptide. Sequence analysis was by automated Edman degradation (——), or by manual Edman degradation (——). Carboxy-terminal analyses were by carboxypeptidase B digestion(←——).

TABLE V: Homology among the Variable Regions (Positions 0 to 107), Variable Region Frameworks (Positions 0 to 29, 34 to 88, and 98 to 107), and Variable Region Complementarity-Determining Regions (Positions 30 to 32 ff and 89 to 97 ff) of Rabbit Antipneumococcal Type III Polysaccharide Antibody Light Chains.

light chain[a]	% homology														
	entire variable region					variable region framework					variable region complementarity regions				
3368		76	75	73	75		84	83	80	82		8	8	17	17
3374			79	78	80			86	85	85			17	17	33
BS-1				93	84				97	90				58	42
BS-5					81					86					42
K25															
	3368	3374	BS-1	BS-5	K25	3368	3374	BS-1	BS-5	K25	3368	3374	BS-1	BS-5	K25

[a] Sequence data for BS-1, BS-5, and K25 are from Jaton (1974a,b, 1975); the 3374 sequence is from Margolies et al. (1975).

by classical methods would require analysis of multiple overlap peptides. As the hypervariable regions of antibody light chains are characterized by marked variations in chain length (Haber et al., 1977) the sequence is best assessed by Edman degradation encompassing these segments of the chain.

Rabbit *b4* light chains contain highly conserved (invariant to date) arginine residues at positions 61 and 211 (Margolies et al., 1975). Thus, large peptides that are suitable for automated degradation can be reproducibly obtained by restricting tryptic cleavage to peptide bonds through modification of lysines with an appropriate reagent (Freedlender & Haber, 1972). In the present study, citraconic anhydride was selected for the modification of lysines because of the ability to deblock under relatively mild conditions (Atassi & Habeeb, 1972), thus permitting additional tryptic digestion of deblocked peptides. The fluorescamine assay provides a reliable and rapid on-line method for assaying complete modification of primary amines that requires minimal expenditure of sample.

We previously reported a comparison of light chain variable region amino acid sequences from antibodies elicited in rabbits (Margolies et al., 1975). Even among the light chain variable regions of antibodies specific for the same simple antigens, striking differences in both sequence and length are found in the complementarity-determining regions (positions 30 to 33 and positions 89 to 98) of the light chain. Although less marked, differences in sequence and length are also found in the nonbinding site (framework) portion of the light chain variable region (Margolies et al., 1975; Cannon et al., 1976). In Table V the homology among the frameworks and complementarity-determining regions of five antipneumococcal type III polysaccharide antibody light chain variable regions are recorded. Although the complementarity-determining regions constitute less than 12% of the variable region length, these short segments contain from 33% to 62% of the residue differences among the variable regions of the five antipneumococcal type III polysaccharide antibody light chains. In addition to differing from the other light chains by 10 or 11 residues for the 12 complementarity region positions, the 3368 light chain also demonstrates an insertion of three residues relative to the BS-1, BS-5, K25, and 3374 light chains in the complementarity region at positions 89 to 98 (in Figure 5 these residues are placed in positions 97A, 97B, and 97C).

While immunization with the pneumococcal type III vaccine often results in production of large amounts of one or a few antibody components by individual rabbits in an outbred population (Chen et al., 1973), each animal utilizes a unique combining site and framework structure to respond to this seemingly simple antigen. This paradox can be explained by invoking extensive polymorphisms within the Ig variable region genome of an outbred population. However, alternative explanations seem more likely (Haber et al., 1976) and include: (a) regulatory expression of a large number of germ-line variable region structural genes; or (b) somatic mutation of a relatively smaller number of germ-line variable regions genes to yield variable regions with specificity for the type III polysaccharide antigen. By alternative (a) above, the germ-line of each rabbit would contain structural genes for all the variable regions found in the population; e.g., the 3368 rabbit possesses structural genes for the BS-1, BS-5, K25, and 3374 light chain variable regions. However, the expression of these genes must be regulated such that, at least for the type III polysaccharide specificity, only one or a few genes are expressed. The somatic hypothesis, above, requires that the type III polysaccharide specificity is a somatically acquired specificity (Cohn et al., 1974). Resolution of these concepts or amplification of other explanations will require additional variable region structures of elicited antibodies from both outbred and inbred animals.

Acknowledgments

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Purification and Characterization of a Colony Stimulating Factor from Human Lung†

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ABSTRACT: Conditioned medium prepared from human autopsy lung tissue contains high level activity of colony stimulating factor which stimulates granulocytes and macrophage colony formation in both mouse and human bone marrow. The lung colony stimulating factor has been purified about 2250-fold by methods including hydroxylapatite chromatography, preparative gel electrophoresis, preparative isoelectric focusing, and gel filtration chromatography. The final specific activity was 2.7×10^6 units/mg. The purified factor has a molecular weight of 41 000 as determined by gel filtration. It is stable at the pH range of 6.5–10 and at 56 °C for 30 min but sensitive to protease digestion and periodate oxidation. On polyacrylamide gel electrophoresis, it migrates in the α-globulin post-albumin region. Upon isoelectrofocusing lung colony stimulating factor appears heterogeneous with isoelectric points of 3.7–4.3. Treatment with neuraminidase did not affect its activity, but caused a change in electrophoretic mobility and isoelectric point. Antibody produced by immunizing rabbits with partially purified lung colony stimulating factor exerted strong inhibitory activity on the factor from lung as well as on colony stimulating factor from other human sources including serum, urine, and placenta.

During the past decade, the study of the mechanism involved in the regulation of granulopoiesis has been facilitated by the introduction of in vitro assay techniques. A method for cloning bone marrow cells in soft agar cultures was reported, independently, by Pluznik & Sachs (1965) and Bradley & Metcalf (1966). When colony forming cells in the marrow are grown in this assay system, they proliferate to form colonies composed of macrophage and/or granulocytic cells. The formation and development of these colonies depend on the presence of an inducing substance which has been called colony stimulating factor or CSF[1] (Metcalf & Foster, 1967; Paran & Sachs, 1968). Hence the target cell for CSF has been termed CFU-C or colony forming unit-CSF dependent. Because of its essential role in the formation of macrophage and granulocytic colonies in vitro, CSF has been postulated to be the primary regulator of myeloid production in vivo (Metcalf, 1973).

CSF can be isolated from human serum (Foster et al., 1968) and urine (Stanley & Metcalf, 1969) and a variety of human tissues such as lung (Fojo, 1977; Fojo et al., 1977), placenta (Ratzan & Yunis, 1974), and peripheral leukocytes (Iscove et al., 1971). Medium conditioned by these tissues will stimulate granulopoiesis in vitro.

CSF has been partially purified from medium conditioned by human peripheral leukocytes (Price et al., 1975) and human placenta (Burgess et al., 1977b). It has been purified to apparent homogeneity from human urine (Stanley & Metcalf, 1973), mouse lung (Burgess et al., 1977a), and cultured mouse cells (Stanley & Heard, 1977; Guez & Sachs, 1973).

Clearly the apparent role of CSF in the control of granulopoiesis and its disorders has added great impetus to the study of this poietin. In spite of the wide distribution of CSF in various tissues, the sources of human CSF have been limited; thus, most of the studies dealing with in vitro human bone marrow culture have utilized peripheral leukocyte-feeder layer as a source of CSF. Curiously human urinary CSF stimulates CFU-C growth in mouse but is weakly active in human bone marrow (Metcalf, 1974). We have found that serum free conditioned medium prepared from autopsy human lung provides a rich source of CSF which stimulates granulocytic and macrophage growth from both mouse and human marrow (Fojo et al., 1977). In this paper we report further purification

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‡ Howard Hughes Investigator.

[1] Abbreviations used: CSF, colony stimulating factor, CFU-C, colony forming unit-CSF dependent; HLCM, human lung conditioned medium; SF-DME, serum-free Dulbecco's modified Eagle's media.

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