$$(V_o/V_i)_{max} = 10^{-\Delta pH} + 2(V_{m_i}/V_{m_o}) \quad (13)$$

where $(V_o/V_i)_{max}$ is the value for maximum sensitivity. Apparently, the choice of vesicle concentration for maximum sensitivity depends on the range of ΔpH to be estimated. This is evident from the plots in Figure 6 where S is just the slope of the curve at any point.

The technique described in this report for estimating transmembrane pH gradients is similar in principle to that described by Deamer et al. (1972) using the fluorescent amine 9-aminoacridine. The charged form of the acridine is believed to be impermeable and concentration gradients of acridine develop across membranes in the presence of pH gradients for the same reasons that gradients of I develop under similar conditions. However, the experimental detection of the gradient is fundamentally different in the two approaches. For the acridine, quenching of fluorescence is used to monitor the internal dye concentration while equilibrium membrane binding is used to monitor internal concentrations of I. The spin label approach thus does not rely on probe–probe interactions and in principle may be used with very low concentrations of I, on the order of a few molecules per vesicle. In addition, this technique should prove to be advantageous in the study of transmembrane pH gradients in vesicles containing light sensitive proteins such as rhodopsin.

Acknowledgments

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Isolation of an Active Variable-Domain Fragment from a Homogeneous Rabbit Antibody Heavy Chain. Physiochemical and Immunological Properties†

Mario S. Rosemblatt‡ and Edgar Haber*,§

ABSTRACT: A fragment corresponding to most of the variable domain of the rabbit heavy chain (V_H) was obtained by tryptic digestion of the mildly reduced and aminoethylated heavy chain from rabbit antibody 3T72. The domain size peptide was purified by gel filtration and shown to extend between residues 11 (Leu) and 122 (Lys) of the heavy chain by sequence analysis. The molecular size of the fragment (approximately 11 000) was determined by gel filtration under denaturing conditions. Under nondenaturing conditions (20 mM sodium acetate, pH 5.5, 0.1 M NaCl), however, the fragment exists as a mixture of monomeric and dimeric species. The variable-domain fragment retains the allotypic determinants of the heavy chain (a_1), as shown by double diffusion on agar plates and radioimmunoassay. Upon recombination of the heavy-chain variable-domain fragment with its homologous light chain, partial recovery of specific binding activity toward the SIII polysaccharide antigen was demonstrated. The method reported here is reproducible (with yields varying between 40 and 60%) and may provide a general method for obtaining the variable region of the heavy chain for antigen binding and allotypic and amino acid sequence studies.

Considerable evidence now supports the concept initially proposed by Edelman (1970) that antibodies are comprised of compact globular domains of approximately 110–120 amino acid residues in length. These independently folded homology regions are interconnected by extended portions of the polypeptide chain. The globular domains have been shown to be highly resistant to endopeptidase attack, while the interdomain connecting peptides are amenable, under controlled conditions, to hydrolysis by various endopeptidases (Gall and D'Eustachio, 1972).

Inbar et al. (1972) reported the isolation of a fragment that comprises the variable domains of both H[1] and L chains from

† From the Department of Medicine, Harvard Medical School and the Massachusetts General Hospital, Boston, Massachusetts 02114. *Received April 11, 1978.* This work was supported by United States Public Health Service Grant AI04967.

‡ Present address: Department of Muscle Research, Boston Biomedical Research Institute, Boston, Mass. 02114.

§ Present address: Cardiac Unit, Massachusetts General Hospital, Boston, Mass. 02114.

[1] Abbreviations used: PBS, 10 mM phosphate buffer, pH 7.4, 0.15 M NaCl; H, heavy; L, light; DEAE, diethylaminoethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid; TPCK, (1-tosylamido-2-phenyl)ethyl chloromethyl ketone; Pth, phenylthiohydantoin; DTT, dithiothreitol.

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the mouse IgA myeloma protein MOPC 315 which retained the ligand-binding activity of the original protein. Sharon and Givol (1976) recombined the variable domain from the light chain with the heavy chain to obtain a fully active Fv fragment after papain digestion. Kakimoto and Onoue (1973) isolated a peptic fragment comprised of the variable domains of H and L chains from human IgM myeloma Hi, and Lin and Putnam (1977) prepared Fv from five human IgM proteins. These studies, however, were difficult to extrapolate to other proteins.

The isolation of a fragment from a nonimmune mixture of rabbit H chains comprising the variable one-quarter of the molecule containing the allotypic *a* locus determinants has also been reported (Mole et al., 1975). Evidence of either ligand-binding activity or identification of amino or carboxy termini of this peptide was not reported.

We present in this report the preparation and purification of a variable-domain fragment from a homogeneous rabbit type III pneumococcal polysaccharide specific antibody H chain. The peptide retains the a_1 allotype determinants of the H chain, and on recombination with homologous L chain it exhibits partial recovery of antigen-binding activity.

Materials and Methods

General. Preparation of the formalinized type III pneumococcal vaccine and type III polysaccharide was previously described (Pincus et al., 1970). Immunization schedule of rabbit 3T72, screening of sera for restricted components by cellulose acetate electrophoresis, and analysis of the number of antibody components produced by rabbit 3T72 by alkaline urea–acrylamide gel electrophoresis of the fully reduced and alkylated antibody were as previously detailed by Chen et al. (1973).

Assay of protein and peptide fractions with fluorescamine (Roche Diagnostics, N.J.) was performed by adding an aliquot of the sample (10–50 μL) to 0.3 mL of a 50 mM sodium borate buffer, pH 8.2, followed by the addition of 0.2 mL of a fluorescamine solution (30 mg/100 mL) in acetone. Fluorescence was measured at 475 nm with an excitation wavelength of 390 nm.

Amino acid composition was determined in a Beckman 121 analyzer after acid hydrolysis at 110 °C in vacuo. Labeling of proteins with ^{125}I (New England Nuclear, Boston, Mass.) was performed by the method of Marchalonis (1969). Binding affinities of 3T72 antibody and the H–L chains recombinant toward a [^{3}H]hexasaccharide fragment of the type III polysaccharide (kindly donated by Dr. Francis Chen) were assessed by equilibrium dialysis to be 0.63×10^7 and 0.49×10^7 mol^{-1}, respectively (Kimball, 1972). Molecular size determination by gel-filtration chromatography was done essentially as described by Andrews (1965).

Determination of a and b Locus Allotypes. The *b* locus allotype of 3T72 antibody and L chain as well as the *a* locus allotype of 3T72 H chain and H chain fragments were determined by immunodiffusion (Ouchterlony, 1953). Immune precipitation of 3T72 L chain with *N*-ethyl chloroformate cross-linked anti-*b4* serum (kindly donated by Dr. Charles Todd) was done essentially as described by Tosi and Landucci-Tosi (1973).

Antibody-Binding Assay. The binding of anti-SIII antibody 3T72, its isolated H chain, and the H–L chain recombinant to [^{125}I]lysozyme-SIII (kindly donated by Dr. E. Cannon) was performed as follows.

Doubling serial dilutions of a known amount of antibody (as determined by amino acid analysis) were carried out in PBS.[1] Fifty microliters of each dilution was added to 1.5-mL conical polypropylene tubes containing 50 μL of a PBS solution of [^{125}I]lysozyme-SIII (10 000 cpm/50 μL) and 50 μL of a solution of preimmune rabbit IgG (1 mg/mL). The solutions were mixed, incubated at room temperature for 2 h, and stored at 4 °C overnight. Separation of bound and free antigen was achieved by the addition to each tube of 50 μL of goat anti-rabbit serum (an amount sufficient to precipitate all the rabbit IgG present in the assay mixture). After a 2-h incubation period at room temperature, the tubes were centrifuged on a Beckman microfuge for 5 min. A 100-μL aliquot of the supernatant was transferred to another tube, and both tubes were counted in a gamma scintillation counter. A maximum of 69% binding was obtained with antibody-containing tubes with a nonspecific (blank) binding of less than 10%.

Studies designed to determine the binding of antigen to other fractions derived from 3T72 antibody were done as described above, except that bound and free antigens were separated in the case of L chains by the use of an anti-*b4* allotypic antiserum, and in the case of V_H and its recombinant with L chain by the addition of anti-a_1 allotypic antisera.

Concentrating the Protein Fractions. Protein fractions destined for sequence analysis were concentrated by lyophilization. Fractions to be used in the binding studies were concentrated either by ultrafiltration using a Diaflo membrane (Amicon Corp., Lexington, Mass.) or in the instance of V_H fractions with a 3000 WM cutoff dialysis membrane (Arthur H. Thomas, Philadelphia, Pa.) against Sephadex G-25 (Pharmacia).

Fractionation of 3T72 Serum. Weekly bleedings showing a restricted response as judged by cellulose acetate electrophoresis and containing between 15 and 20 mg of IgG were dialyzed against 1 mM potassium phosphate buffer, pH 7.45, and applied to a DEAE-cellulose (DE-52 Whatman, England) column preequilibrated with the same buffer. After a two-column volume wash with the starting buffer, a linear gradient between 10 mM and 0.5 M of the same buffer was started. Those antibody fractions showing a single light and heavy chain in alkaline urea gels were pooled, concentrated, and dialyzed against the buffer utilized for mild reduction and aminoethylation (0.5 M Tris-HCl, pH 8.4, 5 mM EDTA).

Preparation of Aminoethylated H Chains. To a final concentration of antibody between 10 and 20 mg/mL in the above-mentioned buffer, DTT was added to a final concentration of 20 mM and the reaction allowed to proceed at room temperature for 2 h under N_2. Aminoethylation of the reduced disulfide bonds was accomplished by three consecutive additions of ethylenimine (ICN Pharmaceuticals, Plainview, N.Y.) at 10-min intervals to a final concentration 20% in molar excess to that of free sulfhydryl groups. Fifteen minutes after the last addition, the protein was dialyzed against distilled H_2O and then against 5 M guanidine hydrochloride–0.1 M sodium acetate, pH 5.5.

The aminoethylated heavy (H) and light (L) chains were separated by gel filtration on a Sephadex G-100 column equilibrated in 5 M guanidine hydrochloride–0.1 M sodium acetate, pH 5.5. The isolated chains were dialyzed first against distilled H_2O, adjusted to pH 5.5 with acetic acid, and then against 20 mM sodium acetate, pH 5.5. The L chain was kept frozen at −20 °C for further use; H chains were concentrated by ultrafiltration. During concentration, some H-chain material precipitated. The precipitated material was resolubilized by dialysis of the concentrated H-chain suspension against 5 M guanidine hydrochloride–0.1 M sodium acetate, pH 5.5, until completely dissolved, then extensively against 20 mM sodium acetate, pH 5.5, and finally against distilled H_2O adjusted to pH 5.5. The H chains obtained in this manner were

fully soluble and immediately used for tryptic digestion. Carboxymethylated (with iodoacetamide) H chains were prepared (as previously described, Chen et al., 1973) and used in comparative experiments.

Tryptic Digestion of the Aminoethylated and Carboxymethylated H Chains. Aminoethylated or carboxymethylated H chains dissolved in H_2O (pH 5.5) at a final concentration of approximately 5 mg/mL were placed in a titration vessel (under N_2) and brought slowly to pH 8.5 by addition of 0.1 N NaOH. The H chain precipitated as a fine suspension. TPCK-trypsin (Worthington Biochemicals Corp., N.J.) was dissolved in 1 mM HCl and added as a single aliquot at a 1:20 weight ratio. The reaction was kept under constant mixing at room temperature and the pH maintained by the addition of 0.1 N NaOH from an automatic burette. During the first few minutes of trypsin action, the pH dropped to 7.0–7.5 while the automatic burette was adding the NaOH at its fastest rate. The reaction was allowed to continue at room temperature for 15 to 20 min and stopped by lowering the pH to 3.5 with acetic acid.

Separation of the H-Chain Tryptic Peptides. The clear solution (approximately 5.0 mL) obtained after tryptic digestion of the aminoethylated H chain was made 5 M in guanidine hydrchloride (by addition of solid guanidine hydrochloride) and submitted to gel filtration on a Sephadex G-100 column (2.6 × 95 cm) equilibrated with 5 M guanidine hydrochloride, 0.1 M acetic acid. Fractions from each peak were pooled, dialyzed against 0.1 M acetic acid, using a 3000-dalton cutoff membrane (A. Thomas, Philadelphia, Pa.), concentrated, and kept frozen until used. Chromatography was carried out under identical conditions of the peptides obtained after digestion of the carboxymethylated H chain.

Amino Terminal Sequence Analysis of V_H. Automated Edman degradation of 50 to 150 nmol (as determined by amino acid analysis) of fully reduced and alkylated V_H was carried out in a modified Beckman 890B sequencer using a 0.1 M Quadrol program (Brauer et al., 1975). Amino-terminal yields varied between 50 and 80%; the average repetitive yield was 94%. The methods for the identification of the Pth-amino acids by gas–liquid chromatography and by thin-layer chromatography have previously been described (Brauer et al., 1975). Arginine was identified on a Beckman 121 analyzer after back-hydrolysis with HI (Smithies et al., 1971).

The C-terminal residue was determined after incubating 120 nmol of fully reduced and carboxymethylated V_H with a mixture of carboxypeptidases A and B and withdrawing aliquots at known times (Ambler, 1967).

Recombination Experiments. Recombination of H chain with homologous L chain was done at a molar ratio of 1:2, while recombination of V_H with its homologous L chain was done at a 1:1 molar ratio, according to the general method of Bjork and Tanford (1971), except that a 3000-dalton cutoff membrane was used on recombination experiments involving V_H. The yield of H–L chain recombinant was assumed to be 95% (Bjork and Tanford, 1971; Huser et al., 1975). To determine the extent of recombination between V_H and its homologous L chain, an aliquot of the recombinant mixture (0.1 mL) was labeled with ^{125}I using the method of Marchalonis (1969) and submitted to gel filtration on a calibrated Sephadex G-100 column.

Results

Two 5-mL aliquots of antiserum from two consecutive weekly bleedings of rabbit 3T72 were pooled and fractionated on DEAE-cellulose. The major peak in the globulin fraction showed a single band for both H and L chains on disc gel electrophoresis. This antibody had a unique amino acid sequence of the L and H chain framework segments, although minor heterogeneity was observed in hypervariable regions (Margolies et al., 1977; Haber et al., 1977).

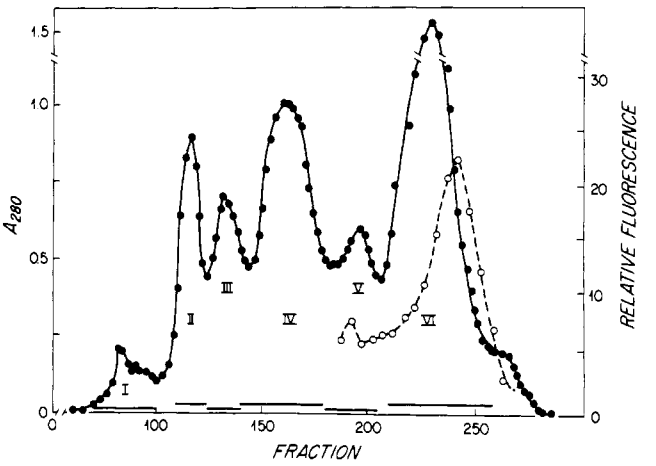

FIGURE 1: Gel-filtration chromatography of a tryptic digest of mildly reduced and aminoethylated 3T72 heavy chain. One hundred milligrams of a heavy-chain tryptic digest was dissolved in 5 mL of 5 M Gdn-HCl, 0.1 M acetic acid, centrifuged, and applied to a Sephadex G-100 column (2.5 × 100 cm) equilibrated with the same buffer. Two-milliliter fractions were collected. Fractions substantially retarded by the column (representing small peptides) were assayed with fluorescamine. The fractions were pooled as indicated: A_{280} (●–●); relative fluorescence (O- - -O).

This fraction was concentrated by ultrafiltration and its aminoethylated H and L chains were prepared. Following tryptic digestion of 100 mg of H chain, peptides were separated by gel filtration on Sephadex G-100 in acid–guanidine as shown in Figure 1. Fractions I, II, and III were pooled and dialyzed against 0.1 M acetic acid using a conventional dialysis membrane. Fractions IV and V were dialyzed against 0.1 M acetic acid using a 3000-dalton cutoff membrane. Aliquots from each one of the fractions were lyophilized and rechromatographed under denaturing conditions (guanidine hydrochloride, 0.1 M acetic acid) after full reduction and alkylation. The peak eluting closest to the void volume of the column was pooled and submitted to amino acid analysis. Gel filtration under identical conditions of a tryptic digest of mildly reduced carboxymethylated H chain gave a quite different pattern; yields of fraction IV were considerably lower, and this digest was thus not studied further. Figure 2 shows the chromatographic profile of a fully reduced and alkylated aliquot obtained from pool IV of Figure 1. Seventy-four percent of the material appears as a single symmetrical peak and probably represents the intact polypeptide chain. Smaller peptides are probably the result of internal cleavages within this chain, initially held together by disulfide bonds. Table I compares the compositions of each fraction in Figure 1 with the amino acid content of known portions of the rabbit H-chain sequence and of trypsin (see references in Table I). Fraction III seems to be trypsin.

Since the amino acid composition of fraction IV (Figure 1) agreed with the composition of the variable domain of the H chain lacking the first ten residues, an aliquot (50 nmol) of fully reduced and alkylated pool IV was submitted to automatic Edman degradation for 28 cycles. A single amino acid sequence was obtained through the region identical with a segment of the first variable region of this heavy chain previously reported (Margolies et al., 1977). This is shown in Figure 3.

The N-terminal sequence determination together with amino acid composition and size of the fragment (see below)

TABLE I: Amino Acid Compositions of Fractions I to V from Gel Filtration of the Tryptic Digest of Aminoethylated H Chain[a] (Figure 1).

	fraction I	Hch[b]	fraction II	res 256 to end[c]	fraction III	trypsin	fraction IV	res 11 to 122	fraction V	res 133 to 204
Lys	24.9	24	13.6	12	16.1	15	5.8	7	3.0	3
His	4.2	5	2.4	3	3.1	3	1.3	1	0.9	1
Arg	17.2	17	6.7	7	2.4	2	3.6	4	1.9	2
Asp	34.2	34	16.3	17	25.6	26	9.3	8	6.3	6
Thr[d]	43.3	45	13.4	14	11.1	10	14.4	16	13.0	14
Ser[d]	44.3	45	18.8	18	33.5	34	14.6	13	14.9	14
Glu	29.6	36	20.5	21	15.1	14	9.4	9	5.8	4
Pro	44.8	46	17.9	15	9.1	8	7.2	7	7.4	8
Gly	33.6	31	13.1	9	26.4	25	10.7	11	6.7	6
Ala	19.0	19	8.1	8	14.6	14	6.7	7	2.7	3
Val	39.2	40	14.1	15	18.2	18	10.1	10	9.5	12
Met	6.3	6	1.9	2	1.6	2	2.1	2	0.6	1
Ile	14.3	14	7.4	8	13.9	15	3.9	4	1.6	1
Leu	28.1	28	10.4	10	14.9	14	9.3	9	6.1	5
Tyr	11.5	13	5.2	6	9.0	10	5.3	5	2.4	2
Phe	11.1	12	5.7	6	3.4	3	3.4	3	1.9	1

[a] No Trp analysis was performed. Lower than expected Cys values were found in most cases and are not reported here. This could be due to excessive oxidation using acid hydrolysis. [b] This amino acid composition is that of the original H chain used in these studies. [c] This amino acid composition was calculated by subtracting the amino acid composition of the known sequences of 1-10 (Wilkinson, 1969) and 224 to 256 from the amino acid composition of fragment C-1 obtained after CNBr cleavage of the entire H chain. [d] Values for these residues were obtained from 24-h hydrolysis.

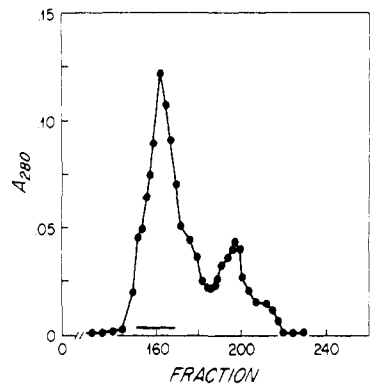

FIGURE 2: Gel-filtration chromatography of a fully reduced and alkylated V_H. An aliquot corresponding to approximately 10% of fraction IV from Figure 1 was fully reduced and alkylated, dissolved in 5 M Gdn-HCl, 0.1 M acetic acid, and rechromatographed on the same column used in Figure 1. Two-milliliter fractions were collected. The fraction eluting earliest in the column and having an elution volume corresponding to that of fraction IV of Figure 1 was pooled and submitted to amino acid analysis and automatic Edman degradation.

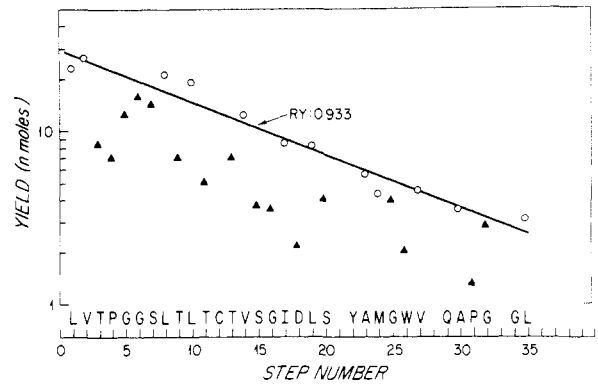

FIGURE 3: The partial amino-terminal sequence (positions 11 to 45) of fully reduced and alkylated 3T72 V_H is shown on the abscissa. Yields of Pth-amino acids determined by gas-liquid chromatography are shown on the ordinate. In this sequenator run, 50 nmol of V_H was used. Yields on stable Pth-amino acids are indicated by circles. Less stable Pth-amino acids are indicated by triangles. The initial yield was 50% and the repetitive yield was 93%. The blanks at positions 28 and 33 indicate uncertain identification. Position 21 is *S*-carboxymethylcysteine as determined by thin-layer chromatography.

suggested that the polypeptide extended from Leu at position 11 to Lys at position 122 of the H chain. Studies performed with carboxypeptidase A and B showed Lys as the C-terminal residue with no aminoethylcysteine present after 4 h of digestion.

The molecular size of V_H was determined by molecular sieving on a G-100 column both under denaturing and nondenaturing conditions. A single symmetrical peak with an apparent molecular weight of 11 000 was obtained for V_H under denaturing conditions. Under nondenaturing conditions, 20 mM sodium acetate, 0.1 M NaCl (the same buffer used for recombination studies), two peaks could be distinguished (Figure 4), one with an apparent molecular weight of 11 000 and a larger size fragment having a molecular weight of 22 300, possibly representing a dimeric form of V_H.

The determinants that define the *a* group allotypes of the rabbit H chain are probably located within the amino-terminal fourth of the molecule (Feinstein, 1963; Mole et al., 1975; Margolies et al., 1977). The V_H fragment isolated by the procedure outlined in this paper can be demonstrated to retain the H-chain allotypic determinant present in the original antibody as shown by two lines of evidence: (a) double diffusion and (b) by antigen precipitation. The results of double-diffusion experiments can be seen in Figure 5. The presence of a_1 allotypic determinants on V_H is also corroborated by a radioimmunoassay developed in order to assess the antigen-binding capacity of V_H (see below). In this assay, precipitation of the V_H-SIII complex (the SIII antigen coupled to [^{125}I]lysozyme, see Methods) can be accomplished by an anti-a_1 antisera, indicating the presence of a_1 allotypic determinants on the V_H fragment.

To determine the antigen-binding activity of isolated V_H, a radioimmunoassay was developed that would permit assessment of the relative activities of V_H and its recombinant product with the L chain as compared to the binding activity

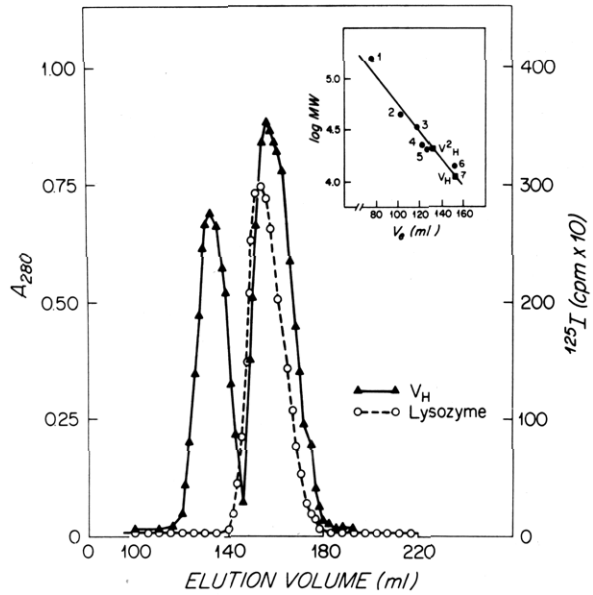

FIGURE 4: Gel-filtration chromatography of V_H under nondenaturing conditions. A 100-μg sample of mildly reduced and alkylated V_H was labeled with ^{125}I, dialyzed against 20 mM sodium acetate buffer, pH 5.5, containing 0.1 M NaCl, and applied to a precalibrated Sephadex G-100 column (1.6 × 100 cm) equilibrated with the same buffer. A 5-mg sample of lysozyme (mol wt 14 000) was cochromatographed with V_H. Fractions (1.0 mL) were collected and assayed for radioactivity (▲-▲) and A_{280} (O-O). The areas under the radioactive peaks were evaluated to be 41% for the first peak and 59% for the second peak. The insert shows the relationship between the elution volume and the molecular size of the V_H peaks. The standards used are 1, rabbit γG; 2, light-chain dimer; 3, β-lactoglobulin; 4, light chain; 5, chymotrypsin; 6, lysozyme; 7, ribonuclease. V_H and V_H^2 (■) represent the monomer and dimer forms of V_H, respectively.

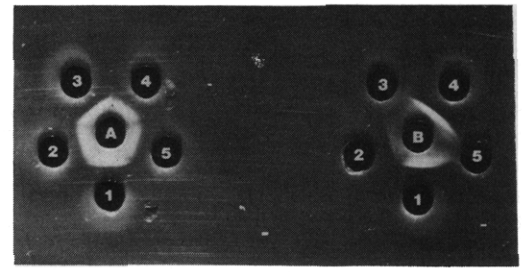

FIGURE 5: Allotype determination of 3T72 antibody and its tryptic fragments by immunodiffusion. The center wells of two agar plates were filled with anti-a_1 allotypic antisera. (A) Wells 1 and 4 were filled with 3T72 antibody. Other wells contained: well 2, purified 3T72 heavy chain; well 3, fraction I from Figure 1; well 5, fraction II from Figure 1. (B) Wells 1 and 4 contained 3T72 antibody, other wells contained: well 3, fraction V from Figure 1; well 2, fraction IV; well 5, fraction III from Figure 1.

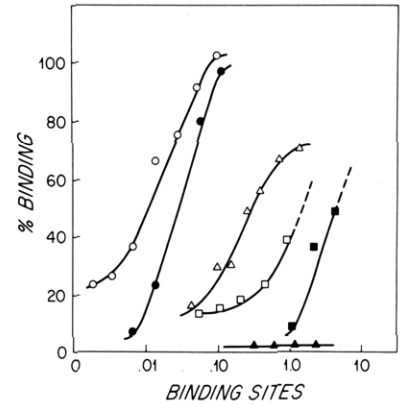

FIGURE 6: Direct binding of [^{125}I]lysozyme-SIII by 3T72-2 antibody (O-O) is compared with the binding of the heavy-light chain recombinant (●-●); V_H-light chain recombinant (△-△), heavy chain (□-□), V_H (■-■), and light chain (▲-▲). Percent binding is corrected for nonspecific binding (<10%) and normalized to the maximal binding of 3T72 antibody. The concentration of potential binding sites was determined from the amino acid composition of each component studied. Antibody and the heavy-light chain recombinants were assumed to have two binding sites per mole while all other components are one binding site per mole. Since V_H was submitted to chromatography in 5 M Gdn-HCl, 0.1 M acetic acid during its preparation, H chain was dialyzed against the same solution overnight before binding studies or recombination experiments.

of isolated H chain and the H-L chain recombinant. The results of such experiments are shown in Figure 6. As expected, the H-L chain recombinant has a binding almost identical to that of the original antibody. Isolated L chains have no binding activity while H chains show an activity (at 50% of binding) of 2.6%. V_H shows binding, after correction for 26% of internally cleaved material (see above), of less than 1% of the original antibody. When V_H was recombined with L chain, a 12% binding activity could be detected for the recombinant. Under optimal conditions, the extent of recombination between H chain and complementary L chain has been determined by several authors to be better than 95% (Bjork and Tanford, 1971; Huser et al., 1975). To determine the reason for the low recovery of activity in the V_H-L chain recombinant, a small aliquot of the recombination mixture was labeled with ^{125}I and chromatographed in a calibrated Sephadex G-100 column. Several peaks were obtained ranging in molecular size from 72 000 to 11 000. This suggested varying degrees of aggregation of the L chain with V_H, as well as possible self-aggregates of both V_H and L.

Discussion

When the rabbit antipneumococcal antibody is mildly reduced and aminoethylated, the isolated H chain should contain aminoethylcysteine at positions 133, 134, 221, and 227. The aminoethylcysteine side chain resembling that of the basic residue Lys has been shown to be susceptible to trypsin cleavage albeit at a reduced rate (Lindley, 1956). These newly introduced points of susceptible cleavage with trypsin facilitated the preparation of V_H, since tryptic cleavage of H chains alkylated with iodoacetamide yielded predominately smaller peptides. Since an appreciable yield of V_H could not be obtained from carboxymethylated chains, it is possible that the initial point of trypsin cleavage occurred at the newly aminoethylated cysteines at positions 133 and/or 134. The peptide produced, however, had Lys as the C-terminal residue with no evidence of aminoethylcysteine. This is confirmed by the absence of aminoethylcysteine on amino acid analysis of the peptide. When these data are considered with the apparent size of the fragment on gel filtration (110 to 120 residues) and the known sequence of the variable region of 3T72 V_H, it is likely that the carboxy terminus of this peptide is Lys-122. Possibly, initial cleavage at aminoethylcysteines-133 and -134 rendered Lys-122 more susceptible to tryptic attack than it would be in the intact H chain. Sequence analysis showed the amino terminus of the peptide to result from a tryptic cleavage at Arg-10 (present in all H chains of the a_1 allotype). An analysis of the three-dimensional folding of V_H (Segal et al., 1974) shows that positions homologous to both Arg-10 and Lys-122 are located in the three-dimensional structure at bends connecting extended segments of polypeptide chains, exposed to the solvent, and thereby more accessible to trypsin attack.

With this variable-domain fragment in hand, we attempted to determine whether or not (a) it would recombine with its

homologous L chain, (b) the recombinant with homologous L chain bound antigen, and (c) it retained determinants that define the *a* group allotype of the rabbit H chain.

Several reports show conflicting results in regard to attempts to recombine isolated variable domains. Karlsson (1972) found that, while an intact L chain is capable of recombining readily with its homologous H chain, L-chain halves (obtained after trypsinolysis) showed no tendency to recombine with intact H chain. Hochman and co-workers (1973) however, demonstrated that isolated inactive V_H and V_L fragments from mouse myeloma protein 315 recombined readily with recovery of 87% of the binding activity of the original protein. Moreover, it has been shown that the peptide linkage at the hinge region between the variable and constant domains of the L chain is not necessary for activity, since rabbit L chains specifically cleaved between Asp-109 and Pro-110 (the switch region) recombined with homologous H chain with full recovery of binding activity toward the SIII polysaccharide antigen (Poulsen et al., 1972).

Under denaturing conditions (5 M guanidine hydrochloride, 0.1 M acetic acid), V_H behaved as a single species with an approximate molecular size of 11 000. Under nondenaturing conditions (20 mM sodium acetate, pH 5.5, 0.1 M NaCl), V_H existed as a mixture of dimer (40%) and monomer (60%). Isolated H chains also behave in a similar manner (Bjork and Tanford, 1971) and the antigen-binding properties of the isolated chains have been attributed by several authors to the formation of a pseudobinding site between the two H-chain variable regions (Painter et al., 1972).

The results presented in this report support prior observations of Inbar et al. (1972) that V_H may be combined with homologous V_L (or in this instance L chain) to produce an active antigen-binding species. The low antigen-binding activity (12%) of the V_H-L recombinant when compared to the H-L recombinant needs to be explained: (a) sequence analysis of the V_H peptide (10–122) does not exclude the presence of significant quantities of peptide 1–122 which would not be detected on Edman degradation because of the presence of an amino-terminal pyrrolidone carboxylic acid. If the sequence 1–10 was essential for antigen-binding activity or recombination with L chain, the presence of a smaller amount of peptide 1–122 may account for all the activity observed; (b) on gel filtration of the V_H-L mixture, a variety of peaks of heterogeneous molecular size were detected. Thus, the V_H-L recombinant is not the only species present, and other recombinants may be inactive.

These studies also confirm the prior observations of Mole et al. (1975) which localize the antigenic determinants of the *a* locus allotype to V_H. For reasons indicated previously, the contribution of residues 1–10 cannot be assessed.

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