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Isolation and Characterization of Structurally Homogeneous Antibodies from Antipneumococcal Sera*

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Abstract. Antibodies of sufficient homogeneity for sequence studies were readily obtained in high concentrations from rabbits immunized with pneumococcal vaccines. By taking advantage of slightly differing immunologic specificity for Type III and Type VIII capsular polysaccharides, an antibody with unique electrophoretic mobility could be isolated from serum containing several distinct antibody components by using appropriate cross-reacting immunoadsorbents. A unique sequence for the N-terminal 11 amino acid residues of the light chain of the antibody was found, in contrast to several sequences in the antibody mixture from which this component was isolated. The sequence of a nonimmune light chain pool demonstrates even greater heterogeneity. Chymotryptic peptide maps of the antibody light chain show two unique cysteine-containing variable region peptides not seen in maps of nonimmune light chain pool of the same allotypic specificity as that of the antibody light chain. The experimental approach described here may provide further insight into the structure–function relationship of several homogeneous antibodies of closely related specificity for the same polysaccharide antigen.

Although much has been learned concerning the primary structure of immunoglobulins from analysis of pathologic *para*-proteins, the relationship between amino acid sequence, three-dimensional structure, and specific antigen binding has been elusive. Two approaches may be employed towards this end: the structural analysis of those *para*-proteins with apparent antigen binding, and the study of antibodies produced through conventional immunization. Many laboratories are pursuing the former course[1–5] but these binding sites may not be relevant as antibody models. For example, the identity of the antigen or hapten with *maximal* binding energy for the site cannot be determined[6] and the participation of the polypeptide chains in forming the binding site seems at variance with that observed in elicited antibodies.[5]

Elicited antibodies proved difficult objects for structural study because of their marked structural and functional heterogeneity (reviewed in ref.[7]). More recently, antibodies of markedly restricted heterogeneity, produced in response to immunization by bacterial vaccines, have been described—first by Krause and co-workers with streptococcal vaccines[8] and later from our laboratory, using pneumococcal vaccines.[9–11] Originally it was believed that only about

10% of rabbits from an outbred population produced a high concentration of one or more electrophoretically restricted antibody components.[10,11] More recently, by using a different immunization schedule, it has become apparent that restricted antibody components can be demonstrated in high concentration in all rabbits immunized at some time during the immune response.[12]

We report the fractionation of antibodies from a single serum containing several distinct components. These components are of sufficient structural simplicity to enable an attack on variable region sequences. Separation is accomplished by taking advantage of slightly differing immunologic specificity for the immunizing antigen. Such fractionation allows for comparison of primary structure of two antibodies to the same antigen in the serum of a single individual, thus minimizing genetic variation and permitting an approach to the question of how many antibodies may be synthesized in response to a single antigen.

Materials and Methods. The growth of Type III and Type VIII pneumococci, the preparation of the corresponding pneumococcal polysaccharides (S3, S8), immunization of rabbits, collection of sera, and quantitative precipitin analysis have been described.[10,11]

The isolation of the type-specific antibodies was performed as follows: An immunoadsorbent was synthesized by coupling either S3 or S8 to bovine serum albumin. These conjugates were then coupled to bromoacetylcellulose (10). 400 mg of the bovine serum albumin-polysaccharide conjugate (S3(8)-BSA) were reacted with 1 g bromoacetylcellulose (dry weight) in 50 ml citrate-phosphate buffer, pH 5.9,[13] and the suspension vigorously stirred for 8 hr at 4°C. More than 95% of the S3 or S8 bovine serum albumin conjugate was adsorbed onto bromoacetylcellulose, as determined by absorbance at 280 nm of the antigen remaining in the supernatant after centrifugation. The subsequent covalent binding of the polysaccharide-albumin conjugate to bromoacetylcellulose was performed as described.[10] Under these altered conditions, the antibody capacity of the immunoadsorbent was increased by a factor of 4, as compared with that previously synthesized.[10]

To isolate the cross-reacting antibody, 50 mg of a 1% antibody solution in phosphate-saline buffer previously isolated with a homologous (S8) immunoadsorbent was mixed with a sufficient quantity of heterologous (S3) adsorbent (100 mg) to completely adsorb the cross-reacting component. After incubation for 30 min at 37°C, the suspension was centrifuged, pellet washed with the buffer, and the supernatant and washings concentrated to the original volume. The washed pellet was then treated with 20 ml of 0.1 N acetic acid for 20 min at 37°C to elute the adsorbed cross-reacting antibody, which was recovered in 80% yield.[10]

Cellulose acetate electrophoresis, full reduction and alkylation, and alkaline urea disc gel electrophoresis were conducted as described.[10,11] The heavy and light polypeptide chains were prepared from the antibodies by mild reduction and alkylation, and were subsequently separated on Sephadex G-100 in 1 M propionic acid.[14]

Nonimmune rabbit γG-globulins from a colony of allotype Aa_1Ab_4 rabbits were isolated by ammonium sulfate precipitation followed by chromatography on DE-52 cellulose.[15]

Chymotryptic digestion was performed for 6 hr at 37°C with an enzyme:substrate ratio of 2:100 in 1% NH_4HCO_3 with fully carboxymethylated protein derivatives. A fully reduced protein sample (5 mg) was alkylated with 2-[^{14}C]iodoacetic acid at pH 8.2 (30 μmol; 0.7 μCi/μmol), to identify the cysteine-containing peptides present in a chymotryptic digest of light chains.

Peptide maps were prepared by the method of Katz *et al.*[16] Chromatography for 24 hr in butanol:water:acetic acid (4:5:1) was followed by electrophoresis in a second dimension in pyridine-acetate buffer, pH 3.6, for one hr at 4000 V in a Gilson Model D Electrophorator. Maps were stained with ninhydrin-collidine. Radioactive peptides were located by exposure of the maps to Kodak No-Screen X-ray film in 14 × 17-inch cassettes

for 72 hr. Radioactive spots were subsequently cut out and counted in Bray's solution[17] in a Packard liquid scintillation counter.

N-terminal analysis of light chains was performed with a protein sequenator developed in this laboratory,[18] based on that described by Edman and Begg.[19] Amino acid sequence determinations by the Edman procedure were done with 5–7 mg of mildly reduced and alkylated light chains. The resulting 3-phenyl-2-thiohydantoin derivatives of the amino acids removed in each successive step were assayed by (1) gas-liquid chromatography by modification[18] of the method of Pisano and Bronzert,[20] and (2) amino acid analysis after conversion of the phenylthiohydantoin derivatives to free amino acids by hydrolysis with 6 N HCl for 24 hr at 150°C.[21] Correction factors for residue loss during acid hydrolysis were applied to all amino acids except serine and threonine which were completely destroyed by this procedure. The latter residues were identified on the high-sensitivity amino acid analyzer after hydriodic acid hydrolysis, which convert phenylthiohydantoin-Ser and phenylthiohydantoin-Thr to Ala and aminobutyric acid, respectively.[22] Quantitative recovery of residues was based primarily on determinations of area inscribed on gas-chromatographic analysis. Those residues which could only be analyzed by amino acid analysis after acid hydrolysis were quantitated with use of correction factors obtained from hydrolysis of phenylthiohydantoin standards. The accuracy of this data is in the range of ±10% for all residues except serine, which is recovered in low and irregular yield.

Results. Antibody from rabbits showing a restricted immune response may contain either one or, more frequently, several electrophoretically distinct antibody components. Figure 1 shows four examples of such sera and their respective isolated antibodies with multiple components.

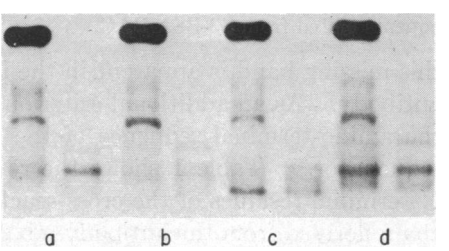

FIG. 1.—Cellulose acetate electrophoretic patterns of various rabbit antisera to Type VIII pneumococci showing a γ-globulin zone with several distinct species. Multiple components are more easily seen in the isolated antibodies. Antisera and isolated antibodies: (*a*) 1302, (*b*) 377, (*c*) 1305, (*d*) 1548. Anode is at the top.

Since cellobiuronic acid is the common subunit to both S3 and S8, some cross-reactions of these anti-S8 antisera with S3 are not unexpected and has been previously demonstrated.[23,24] The extent of cross-reaction of six rabbit antisera containing electrophoretically restricted antibody components, varied from 1 to 97%. The purification of a single component from serum 1305 (43% cross-reactivity) will serve as a typical illustration of the use of such cross-reactions in antibody isolation. The Type VIII specific antibody was isolated from serum by using the homologous S8-BSA-BAC immunoadsorbent; three electrophoretically distinct components were readily observed (Fig. 2). When the isolated antibodies were treated with the heterologous S3-BSA-BAC, the antibody component of slower electrophoretic mobility was almost completely removed from the mixture. Upon subsequent elution with acetic acid it was shown to be a single electrophoretic species (Fig. 2).

The antibody fractions were further examined for charge homogeneity of their light chains by basic disc gel electrophoresis in 8.5 M urea (Fig. 3). The light chains derived from the antibody mixture of the three components shows five distinct bands; those derived from the depleted mixture in which the cross-reacting component was removed show the absence of one slowly moving band;

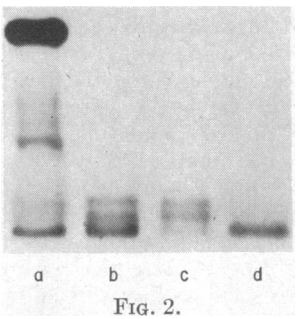

FIG. 2.

FIG. 2.—Cellulose acetate electrophoretic patterns of Type VIII antiserum 1305 and its various isolated antibody components. (*a*) antiserum 1305; (*b*) antibody components isolated from serum with homologous immunoadsorbent S8-BSA-BAC; (*c*) antibody components present in the supernatant after incubation with the cross-reacting immunoadsorbent S3-BSA-BAC; (*d*) cross-reacting antibody components eluted from S3-BSA-BAC.

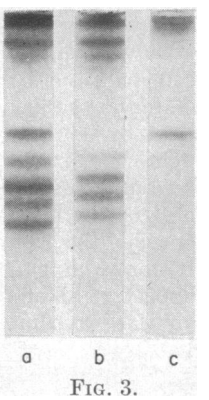

FIG. 3.

FIG. 3.—Disc gel electrophoretic patterns of light chains isolated from antiserum 1305: (*a*) antibody mixture; (*b*) depleted mixture in which the cross-reacting antibody has been removed; (*c*) the cross-reacting antibody component. 300 μg protein was applied. Anode at the bottom.

this missing band is apparent in the light chain isolated from the cross-reacting antibody. As an additional criterion for restriction of structural heterogeneity, the amino-terminal sequence of the light chain was examined by the protein sequenator.[18] Table 1 shows the results of the Edman degradation for the 11 N-terminal residues of the cross-reacting antibody light chain, and for the light chain derived from the antibody mixture. Included also for comparison are the data on the light chain derived from nonimmune γ-globulins from pooled rabbit sera of the same allotypic specificity as the antibody light chains. The Ab_4 light chain pool reflects great sequence heterogeneity. Position 1 shows Ala as the dominant residue (86%) with smaller amounts of Asp (14%). Position 2 and subsequent ones exhibit several major amino acid alternatives. Some of this heterogeneity may be related to deletions or additions at the N-terminus of the light chains and reflects lack of alignment of similar sequences. Met appears at positions 4 and 5, Thr at 4, 5, and 6, Glx at 5, 6, and 7, and Pro at 7, 8, and 9. By homology with human and mouse κ-chains shown at the bottom of Table 1, it is likely that Met is in position 4, Thr at 5, Glx at 6, and Pro at 8 in rabbit light chains with different N-terminal lengths. For example, the appearance of Pro in the mixture at position 7 indicates a chain one residue shorter than the composite human and mouse sequence; at position 8 they are the same length; at position 9 the chain is one residue longer.[25] This cannot, however, explain all the complexity observed in the Ab_4 light chain pool. On the other hand, the light chain from the antibody mixture shows a striking decrease in heterogeneity at positions 1–3. At position 1, Asp accounting for only 14% in the Ab_4 light chain pool has increased to 66% in the antibody mixture. Val

TABLE 1. *Light chain amino-terminal sequences.*

Rabbit light chain	Residue Number 1	2	3	4	5	6	7	8	9	10	11
1305 (Cross-reactive)	Asp	Val	Val	Met	Thr	Glx	Thr	Pro	Ala	Thr	Val
1305 Mixture	Asp 66*	Val	Val	Met 54	Thr 83	Glx 79	Thr 73	Pro 54	Ala 38	Thr 50	Val 60
	Ala 34			Val 19	Leu 17	Thr 21	Val 15	Leu 32	Pro 27	Val 27	Thr 29
				Leu 15			Leu 12	Val 14	Leu 18	Ala 32	Leu 11
				Thr 12					Val 17		
B_4 Pool	Ala 86	Val 62	Val 85	Thr 40	Thr 72	Thr 84	Glx 49	Pro 33	Gly 31	Val 26	Val 38
	Asp 14	Asp 24	Pro 15	Met 35	Met 16	Glx 10	Pro 27	Leu 14	Pro 24	Ala 23	Asp 12
		Ile 14		Val 25	Glx 12		Val 31	Val 19	Ala 20	Pro 18	Pro 15
								Glx 14	Val 12	Leu 18	Leu 15
								Bly 19	Leu 13	Glx 15	Ala 11
											Gly 10
Human and mouse myeloma κ-chains†	Asp	Ile	Val	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu
	Glu	Val	Gln	Leu			Thr		Gly	Thr	
	Lys	Met	Leu	Val					Ala	Phe	
				Ile					Leu		
									Thr		
									Tyr		
									Asx		

* Percentage of residues found. Those present in concentrations less than 10% were excluded.

† Adapted from *Atlas of Protein Sequence and Structure*, ed. M. O. Dayhoff (Silver Spring, Md.: National Biomedical Research Foundation, 1969,) vol. 4.

at positions 2 and 3 is the only residue found in the antibody mixture. Although the mixture in the subsequent positions is complex, there appears to be a dominant sequence. There is evidence for overlap in that Thr appears at position 6 and 7, and Pro at position 8 and 9. However, when the light chain derived from the cross-reacting antibody purified from the mixture is analyzed, a single sequence can be determined for the first 11 amino-terminal residues (Table 1). Background residues are no more prevalent than in a homogeneous Bence-Jones protein[25] run in the same instrument.

The light chain was further examined by peptide mapping. In a sequence of restricted heterogeneity, unique peptide characteristics of the variable region should be demonstrable, whereas in a highly heterogeneous sequence, the relative concentration of these peptides should be too low for clear identification. Figure 4 shows ninhydrin-stained maps of a chymotryptic digest of light chains from the cross-reacting antibody 1305 and from an Ab_4 pool. One can see many peptides of identical mobility in both maps, which probably represent the common regions of the sequence. Some peptides appear unique to the antibody light chain, while some are characteristic of the b_4 pool.

In Figure 4 (*bottom*), the radioautographs of chymotryptic peptide map of ^{14}C alkylated light chains from antibody 1305 is compared to the Ab_4 light chain pool. Only cysteine-containing peptides (as S-carboxymethyl cysteine) are demonstrated by this technique. The fraction of the total radioactivity at the origin varied from 3 to 9%. This relatively small amount of "core" allows for meaningful comparison of the cysteine-containing peptides of several light chain preparations. Tryptic peptide maps of rabbit light chains were unsatisfactory owing to significant amounts of peptides remaining at the origin.

The distribution of cysteine residues between variable and constant regions of the sequence in rabbit light chains has not as yet been determined, nor can one predict the number of peptides on a chymotryptic map in which a single cysteine will appear because of variability of chymotryptic cleavage. Yet it is apparent that there are five major peptides common to the antibody light chain and the Ab_4 light chain pool (Fig. 4). On the other hand, the antibody light chain shows two additional peptides not seen in the pool. These two unique cysteine-containing peptides amounted to 30% of the total radioactivity counted. This finding strongly suggests that these represent the major variable region cys-peptides of antibody 1305.

Discussion. In the experiments reported here several distinct antibody components were fractionated by taking advantage of differing specificity for closely related polysaccharide antigens. By these methods large amounts of antibody sufficiently homogeneous for sequence studies may be obtained from single animals. An isolated antibody (1305) appears to be homogeneous by the following criteria: (1) It is a single electrophoretic species, (2) the sequence of the first 11 N-terminal amino acids of its light chain is unique, and (3) it contains unique variable region peptides not apparent in a nonimmune light chain pool of the same allotypic specificity.

Unequivocal indication of homogeneity, however, will only be obtained through complete amino acid sequence. Our experience with the preparation described here, as well as with others, indicate that a single light chain band on

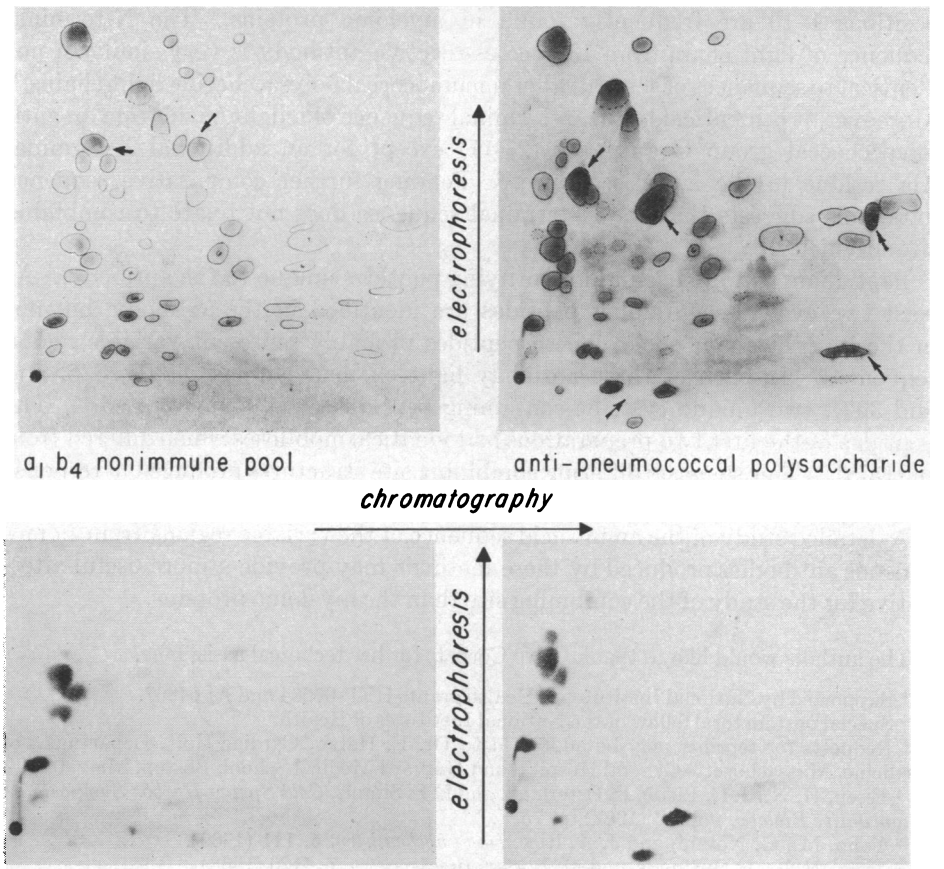

FIG. 4.—Chymotryptic peptide maps of light chains fully reduced and alkylated with [2-^{14}C]-iodoacetic acid. *Top:* Ninhydrin-collidine stain. Arrows indicate peptides unique to each preparation. *Left:* Nonimmune pool. *Right:* Cross-reacting component of antibody 1305. *Bottom:* Corresponding radioautographs. The two unique cysteine-containing variable region peptides are evident in the antibody map.

alkaline urea acrylamide electrophoresis is correlated with a single sequence at the amino terminus, whereas multiple bands indicate mixed sequences. Limitations of the analytic techniques associated with the Edman sequenator, especially the gradual accumulation of background amino acids, do not yet allow for reliable identification of sequences below the 10% level. Nevertheless, it is apparent that the light chain from the cross-reacting component of antibody 1305 has a unique sequence near the N-terminus, in contrast to mixed sequences found in the antibody mixture. Greater sequence heterogeneity was evident in the pool. Some of the complexity of the antibody mixture and of the Ab_4 light chain pool can be attributed to varying polypeptide chain lengths and consequent overlapping sequences.[26]

There seems to be considerable homology between human, mouse, and rabbit κ light chain N-terminal sequences. Residues common to all seem to be Thr 5, Gln 6, and Pro 8. Leu 11, which is almost universal in both mouse and human κ light chains is replaced by Val in the rabbit light chain. All the residues in

positions 1–10 are frequently found in myeloma proteins. The N-terminal sequence of light chain from 1305 cross-reactive antibody is very similar if not identical to sequences of several other pneumococcal polysaccharide light chains.[27] Moreover, it is identical to the N-terminal sequence of a light chain from an anti-streptococcal group C antibody (27-11) except for an additional N-terminal Ala residue in the latter sequence.[26] Pending further comparative sequence data, this suggests that the N-terminal sequence does not relate to combining site specificity.

Peptide maps were useful in identifying peptides unique to the antibody. At least two cysteine-containing peptides are identified in the antibody, but not in the b_4 light chain pool. These peptides probably represent variable region sequences. In each of three antibody light chains examined (1305, 325, and and 392), two unique cysteine-containing peptides were demonstrated.[27] The peptides of the first two preparations had identical mobilities which differed from the third. This suggests differing combining site structures produced in response to the same antigen by different rabbits.

A detailed study of the amino acid sequence of the variable regions from homogeneous antibodies produced by these methods may provide a more useful alternative for the study of the combining site than the myeloma proteins.

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