Variation in the Primary Structure of Antibodies during the Course of Immunization*

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ABSTRACT: Immunization of rabbits with pneumococcal vaccines has been shown to produce antibodies of markedly restricted heterogeneity. The variation in structure of antibodies isolated after each of three courses of immunization from a single rabbit (No. 325) immunized with Type VIII pneumococci is described. The results of these investigations showed that a common amino acid sequence was demonstrable in the N-terminal region in all three antibody light-chain preparations. This was the predominant sequence detected in second-course antibody (>90%); additional residues at several positions, reflecting additional sequences, were found in the first- and third-course antibodies. Peptide maps and corresponding radioautographs of chymotryptic digests of light chains, reduced and alkylated with [2-^{14}C]iodoacetic acid revealed two major cysteine-containing peptides in all three preparations. These peptides originated from the variable region of the polypeptide chain and were not found in digests of light chains obtained from nonantibody immunoglobulin of the same allotype. In the first- and third-course antiboby light chains, these two peptides were present in lower concentrations than in the second-course antibody light chain, and were accompanied by additional cysteine-containing peptides. Guinea pig antisera directed against individual antigenic determinants of the second-course antibody, cross-reacted with first- and third-course antibodies, but not with nonantibody γG-globulin, nor with six other purified rabbit antibodies to Type VIII pneumococci. Thus, three independent experiments confirmed the presence of the same antibody species, in varying concentration, during three courses of immunization.

Tumors of plasma cells or single antibody producing cells may secrete a unique molecular species of immunoglobulin (Nussenzweig *et al.*, 1968; Marchalonis and Nossal, 1968). In contrast, the conventional immune response involves the commitment of many clones of cells with the consequent production of structurally heterogeneous antibody populations (Haber, 1968). It has been difficult to identify constituents of such populations during the course of the immune response because of similarity in size, charge, and antigenic properties of the individual components. However, it has been recently shown that hyperimmunization of rabbits with streptococcal or pneumococcal vaccines resulted in a far less heterogeneous response than that obtained with conventional antigens (Krause, 1970; Haber, 1970). In these systems, electrophoretically restricted antibodies are produced. It has been shown that these antibodies may exhibit allotypic exclusion (Kindt *et al.*, 1970) and individual antigenic specificity (Eichmann *et al.*, 1970), as indications of homogeneity. The availability of these antibodies permitted preliminary determinations to be made of the primary structure of immunoglobulins directed against well-defined antigenic determinants (Hood *et al.*, 1969; Jaton *et al.*, 1970).

This communication presents the N-terminal amino acid sequences and radioautographs of peptide maps of the light chains of purified anti-S8[1] antibodies obtained from a single rabbit after each of three courses of immunization. These studies and the demonstration of individual antigenic specificity of the purified antibody confirmed the presence of the same antibody species, in varying concentration, throughout immunization. A single antibody species predominated after the second course of immunization. Additional antibody species were readily detected after the first and third, but not the second, period of immunization.

Materials and Methods

The methods of culturing Type VIII pneumococci, preparing pneumococcal polysaccharide (S8), immunizing rabbit No. 325, and processing antisera have been described (Pincus *et al.*, 1970). Serum was obtained from rabbit 325 5 days after each of three 1-month courses of immunization; the animal rested for 1 month between courses. Specific antibodies were isolated using a solid immunoadsorbent consisting of S8-azobovine albumin conjugate covalently linked to bromoacetylcellulose (Jaton *et al.*, 1970). Nonantibody γG-globulin was isolated by DEAE-cellulose chromatography from a pool of sera obtained from al b4 homozygous rabbits. Preparation and separation of heavy (H) and light (L) chains was performed as described (Jaton *et al.*, 1970).

Determination of the amino acid sequence of the N-terminal region was performed with a protein sequenator (Waterfield

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[1] The nomenclature for immunoglobulins used is that recommended by the World Health Organization [*Bull. W. H. O. 30*, 447 (1964)]; abbreviations used here that are not listed in *Biochemistry 5*, 1445 (1966), are: S8, soluble Type VIII pneumococcal polysaccharide; C-I, C-II, and C-III, specifically-purified antibodies obtained from serum after a first, second, and third course of immunization; PTH, 3-phenyl-2-thiohydantoin: Cys-peptide, cysteine-containing peptide.

TABLE I: Rabbit Antibody L-Chain Sequences. Residue Positions from the Amino Terminus.[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
I	*ASP*	*VAL*	*VAL*	*MET*	*THR*	*GLX*	*THR*	*PRO*	*ALA*	*SER*	*VAL*	*SER*	*GLX*	*PRO*	*VAL*	*GLY*	*GLY*				
	ALA	PHE	leu	LEU				THR	thr	thr	ser	glx	ALA	ALA	ala	val	thr				
			met	val							thr	thr	thr			ala	ala				
												val									
II	*ASP*	*VAL*	*VAL*	*MET*	*THR*	*GLX*	*THR*	*PRO*	*ALA*	*SER*	*VAL*	*SER*	*GLX*	*PRO*	*VAL*	*GLY*	*GLY*	*THR*	*VAL*	*THR*	*ILE*
III	*ASP*	*VAL*	*VAL*	*MET*	*THR*	*GLX*	*THR*	*PRO*	*ALA*	*SER*	*VAL*										
	ALA	PHE	pro	leu	val	THR	val	val	pro	thr	thr										
				val	leu	val		thr	thr	val	leu										
						leu			val												
Nonanti-	ALA	VAL	VAL	THR	THR	THR	GLX	PRO	GLY	VAL	VAL										
body[b]	asp	asp	pro	MET	met	glx	PRO	val	pro	ala	leu										
b4		ile		VAL	glx		VAL	gly	ala	pro	pro										
								glx	val	leu	ala										
								leu	leu	glx	asp										

[a] Italicized residues represent the unique sequence of C-II. Upper case: = or > than 25% of residues recovered; lower case: 10–24% of residues recovered; residues present at concentration <10% are not listed. [b] From Jaton *et al.* (1970).

et al., 1970a), using 0.1–0.2 μmole of L chains. In the present study, dithiothreitol (5×10^{-4} M) was added to the chlorobutane, since the presence of the reducing agent considerably increased the yield of PTH-amino acids, particularly that of serine (Hermodson *et al.*, 1970). The resulting PTH derivatives of the amino acids removed in each successive step were assayed by (1) gas–liquid chromatography (Pisano and Bronzert, 1969) and (2) amino acid analysis after conversion of the PTH derivatives to free amino acids by hydrolysis with 57% HI for 20 hr at 125°. In this procedure, serine and threonine were identified by amino acid analysis as alanine and β-aminobutyric acid, respectively (D. Gibson and O. Smithies, 1969, personal communication). Methionine, which was completely destroyed by HI hydrolysis, was identified after hydrolysis in 6 N HCl for 24 hr at 150° (Van Orden and Carpenter, 1964). Values reported include corrections for loss during hydrolysis based on those of standard PTH-amino acids.

Full reduction and alkylation of L chains with [2-^{14}C]-iodoacetic acid, chymotryptic digestion, peptide mapping of 1.5 mg of lyophilized digests, radioautography, and determination of radioactivity of each spot were performed as described previously (Jaton *et al.*, 1970).

Detection of individual antigenic specificity of the isolated antibodies was carried out by using guinea pig antisera to rabbit anti-S8 C-II. The method of Henney and Ishizaka (1969) was used to render guinea pigs tolerant to a1 b4 γG-globulin, and to subsequently promote a specific immune response to C-II. Guinea pigs were injected intravenously with 1.0 ml of saline containing 5 mg of γG-globulin. Prior to injection, the γG-globulin solution was centrifuged at 34,000 rpm for 1 hr, followed by sterilization of the supernatant using Millipore filters (0.45 μ). One week later, the intravenous injection of γG-globulin was repeated and 1.0 ml of an emulsion containing 50 μg of C-II protein in 0.5 ml of saline and 0.5 ml of complete Freund's adjuvant was injected into the footpads. This procedure was repeated 1 week later. Two weeks following the second immunization the animals were exsanguinated. Other guinea pigs were immunized with 1 ml of an emulsion consisting of 500 μg of C-II in 0.5 ml of saline and 0.5 ml of complete Freund's adjuvant injected into the footpads. On day 21, an additional 100 μg of C-II in 0.1 ml of saline was injected intradermally in two sites and the animals were exsanguinated on day 28.

Double radial immunodiffusion was performed in 1% Difco Noble agar dissolved in 0.15 M NaCl–0.01 M phosphate buffer, pH 7.4. Single radial immunodiffusion was performed as described by Mancini *et al.* (1965).

Results

Amino Acid Sequence Determination of the N-Terminal Region of Antibody L Chains. Table I shows the results of Edman degradation of C-I, C-II, and C-III L chains, as well as nonantibody b4 L chains. The C-I L chain yielded more than one amino acid residue for most positions in its N-terminal region. In contrast, in the analysis of C-II L chain, a single amino acid comprised more than 90% of the residues found at each of the first 21 positions. The C-III L chain contained two or more amino acid alternatives at each position studied. The unique sequence found for the N-terminal region of C-II L chain accounted for a significant proportion of the C-I and C-III mixed L-chain sequences. Noteworthy is the presence of phenylalanine, an unusual residue at position 2 in C-I, its absence in C-II, and its recurrence in C-III L chains at corresponding positions. This residue is not seen at position 2 in nonantibody b4 L chain. In addition, threonine and serine at position 10 are present in the antibody L chains, but are not seen in the nonantibody L chains examined.

Peptide Mapping of Antibody L Chains. Samples of antibody L chains from C-I, C-II, and C-III, as well as from nonantibody b4 L chain were fully reduced and alkylated with [2-^{14}C]-iodoacetic acid. Chymotryptic digests of these L chains were then subjected to peptide mapping on paper (Jaton *et al.*, 1970). Chymotrypsin was used instead of trypsin, since the former yielded a much smaller amount of insoluble material remaining at the origin (less than 10% of total radioactivity

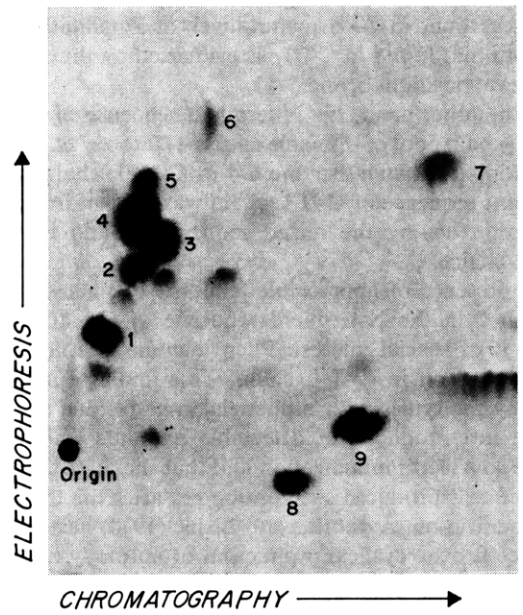

FIGURE 1: Radioautograph of chymotryptic peptide map of C-II L chain fully reduced and alkylated with [2-^{14}C]iodoacetic acid. Peptides of interest are numbered. See text and Table II.

TABLE II: Distribution of Radioactivity among Cysteine-Containing Peptides from Chymotryptic Maps of L Chains (% of Total Radioactivity Counted on Map).

Peptide No.	C-I	C-II	C-III	Non-immune b4
1	9.4	9.5	8.6	9.7
2	5.9	4.3	5.7	5.9
3	5.8	6.1	6.0	6.3
4	13.3	12.8	14.4	11.8
5	3.4	3.5	3.1	1.1
6	1.6	1.7	2.0	1.0
7	4.5	3.3	5.0	4.9
Sum *1–7*	*44*	*41*	*45*	*44*
8	3.1	6.7	3.2	
9	12.8	23.2	12.5	
Sum *8–9*	*16*	*30*	*16*	*0*

applied to the paper). The ninhydrin–collidine stained maps of C-I, C-II, and C-III L chains were difficult to distinguish from one another. Radioautographs, however, showed consistent differences. Figure 1 shows the radioautograph of the C-II L-chain map. Peptides 1–7 were seen in all L-chain preparations studied, including the nonantibody b4 L chain. The sum of the radioactivities of peptides 1–7 accounted for 41–45% of the total radioactivity counted on the map (150,000–250,000 cpm). The relative radioactivities of these peptides were similar in the L-chain preparations listed in Table II. It was inferred that these peptides arose from the constant region of the L chains. Peptides 8 and 9 were seen in the maps of all three antibody L chains, but were not discernible in the nonantibody b4 L-chain map. It is likely that these Cys-peptides were derived from the variable region of the L chain. The sums of the radioactivities counted in spots 8 and 9 were 15.9% of the total activity counted on the paper in C-I, 29.9% in C-II, and 15.7% in C-III (Table II). In the C-I and C-III L chain maps, several additional faint radioactive spots were seen, which were not detected in the C-II L-chain map. These spots may represent additional variable region peptides. The clearly discernible spots listed in Table II comprise 60% of the total radioactivity in C-I, 71% in C-II, 61% in C-III, and 44% in the b4 nonantibody L chains. The remainder of the radioactivity was distributed among multiple faint spots. These faint spots may represent minor chymotryptic cleavage products, or may reflect antibody heterogeneity, *i.e.*, many alternative peptides from the variable region. The latter possibility is supported by the fact that only 44% of the total radioactivity was concentrated in spots numbered 1–7 in the nonantibody L-chain preparation, whereas 71% of the radioactivity was concentrated in spots 1–9 in that antibody preparation, which showed the greatest amino acid sequence homogeneity. The remaining radioactivity in the latter preparation was distributed among 15 other spots, including the origin (5–7%).

Persistence of Individual Antigenic Specificity in C-I, C-II, and C-III. Guinea pig sera obtained early in the course of immunization from animals rendered tolerant to al b4 γG-globulin by intravenous injection and challenged with C-II antibody in complete Freund's adjuvant, yielded precipitin lines in agar gel with C-I, C-II, and C-III but not with al b4 γG-globulin at the same concentrations (100–500 μg). Guinea pig antisera obtained from nontolerant animals injected with C-II and sera from tolerant animals were compared to one another in their reaction with C-II. A precipitin line of partial identity was observed; antisera from nontolerant guinea pigs formed a spur over the precipitin line formed by antigen and antisera from tolerant animals. Following reimmunization of tolerant guinea pigs (see Methods), their antisera were found to react weakly with al b4 γG-globulin. This activity, however, could be removed by absorption with al b4 γG-globulin. Single radial immunodiffusion plates were prepared with the absorbed guinea pig antisera to C-II, as well as with antisera to C-II obtained from "nontolerant" animals (Figure 2A). In this plate, wells filled with C-I, C-II, and C-III (500 μg/ml)

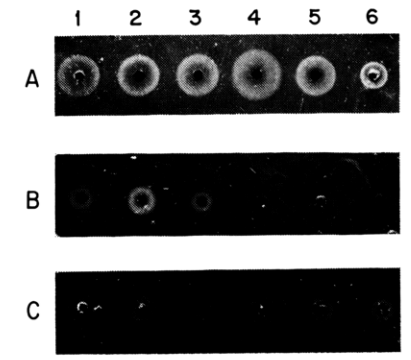

FIGURE 2: Radial immunodiffusion analysis of C-I, C-II, and C-III with guinea pig antisera to C-II. A. Agar contains 0.04 ml of guinea pig antiserum to C-II antibody from "nontolerant" animals (see Methods). Wells 1, 2, and 3 filled with C-I, C-II, and C-III (500 μg/ml), respectively; 4, 5, and 6 with al b4 IgG at 1000 μg/ml, 500 μg/ml, and 250 μg/ml, respectively. B. Agar contains 0.6 ml of absorbed guinea pig antiserum to C-II. Wells filled as in A. C. Agar contains 0.6 ml of absorbed guinea pig antiserum to C-II. Wells filled as in A except that wells 4, 5, and 6 contain C-II at 1000 μg/ml, 500 μg/ml, and 250 μg/ml, respectively.

TABLE III: Radial Immunodiffusion Analyses of Antibodies from the Three Courses of Immunization of Rabbit 325.

Antigens	Protein Concn (μg/ml)	Diameter of Precipitation Ring (mm): Guinea Pig Anti-serum to C-II	Diameter of Precipitation Ring (mm): Absorbed Guinea Pig Anti-serum to C-II	Antibody Molecules Sharing Individual Antigenic Specificity[a] (%)
C-I	500	6.4	4.2	54
C-II	1000		6.6	100
	500	6.4	5.4	100
	250		4.5	
C-III	500	6.4	4.2	45
a1 b4 IgG	1000	8.4	0	
	500	6.4	0	
	250	5.0	0	

[a] Calculated from the plot of log concn C-II *vs.* diameter of precipitation ring.

yielded precipitin rings of equal diameter. Wells filled with the same concentration of a1 b4 nonantibody γG-globulin gave a ring of the same size. In plates prepared with absorbed antisera to C-II, the largest precipitin ring was obtained with C-II; rings of smaller diameter were observed with C-I and C-III (Figure 2B). Precipitin rings were not detected around wells filled with either a1 b4 γG-globulin at concentrations up to 1000 μg/ml (Figure 2B), nor with 6 different electrophoretically restricted anti-S8 antibody preparations (not shown). The diameters of precipitin rings are tabulated in Table III. From the plot of log of concentration of C-II *vs.* diameter of precipitin rings, it was calculated that about 45% of the antibody molecules in C-I and C-III preparations possessed the individual antigenic specificity of C-II.

Discussion

In a previous communication, it was reported that 10 of 116 rabbits injected with Type VIII pneumococcal vaccine showed a restricted immune response (Pincus *et al.*, 1970). Sera obtained from one of these rabbits, No. 325, at intervals after each of three courses of immunization disclosed the presence of one electrophoretically restricted antibody component in each preparation. As previously reported (Pincus *et al.*, 1970), disc gel electrophoretic patterns of L chains isolated from C-I, C-II, and C-III indicated the presence in all three preparations of two predominant fast migrating bands; additional L-chain bands of slower mobility were seen in C-I L chain and C-III L chain. These findings suggested that some of the clones of antibody forming cells may cease to secrete antibody during the course of imminization. At a later time, either these or other antibody secreting clones may resume activity (Waterfield *et al.*, 1970b).

From an examination of the N-terminal sequences of L chains derived from C-I, C-II, and C-III (Table I), it is apparent that both C-I and C-III L chains are structurally heterogeneous, while the C-II L chain appears to have a unique sequence in its N-terminal region. The mixture of sequences found in C-I is quantitatively and qualitatively different from that found in C-III, as evidenced by the amino acid residues at positions 3, and 5–11.

On the other hand, the N-terminal sequence of a nonantibody L-chain pool of the same allotype (Jaton *et al.*, 1970) was more complex than either the C-I or C-III L chain. The predominant sequence of C-II L chain was present in significant proportion among the mixed sequences found in C-I and C-III L chains.

The presence of appreciable amounts of phenylalanine at position 2 in the N-terminal sequence of C-I and C-III L chains is of special interest. Phenylalanine was not detected at this position in C-II L chain. This finding suggests that antibody molecules with a phenylalanine residue at position 2 were not produced in detectable amounts following the second course of immunization and that the same or a similar clone of cells produced such antibodies after the third course of immunization. Avrameas and Leduc (1970) have presented evidence for the cyclical production of antibody by lymphoplasmocyte and have proposed that this cell has the dual function of "memory" and antibody production. It is not known whether such cyclical activity could account for the variation in antibody species observed in the course of the present study.

An alternate interpretation of these data is that the clone producing the major sequence proliferated early during immunization while the other antibody forming clones did not proliferate until after the third course of injections. This is supported by yields of anti-Type VIII antibody of 3.5, 17, 49.5 mg per ml, respectively, after the first, second, and third course of immunization (Pincus *et al.*, 1970) and of 1.7, 16, and 25 mg per ml of the dominant sequence in the three sera. These data are not likely to be distorted appreciably by preferential elution of some antibody species from the immunoadsorbent since absolute recoveries of antibody were 87, 86, and 84%, respectively, in the three antisera.

It is of considerable interest that two previously reported amino-terminal L-chain sequences of rabbit antipolysaccharide are very similar to that of C-II. The amino-terminal L-chain sequence of an S8 antibody (1305, Jaton *et al.*, 1970) was identical with that of 325 C-II from residues 1 to 11, except that at position 10, threonine was substituted for serine. Both antibodies cross-reacted with S3. The two variable region Cys-peptides were identical in mobility on peptide mapping. On the other hand, antiserum directed at individual antigenic determinants of 325 C-II did not react with 1305 antibody in immunodiffusion. This suggests that the sequences are not identical throughout. An anti-group-C streptococcal antibody (R27-11, Hood *et al.*, 1970) has an identical L-chain N-terminal sequence for 17 residues examined, except for an additional alanine residue at the N terminus. Six other L-chain N-terminal sequences of antigroup-C streptococcal antibodies showed differences within the first 4 residues when compared to 325 C-II, 1305, and R27-11 antibody L chains. More extensive sequence determinations are necessary in order to define the relationship of this sequence variability to antibody specificity.

The persistence of antibody molecules of similar or identical structure throughout immunization was also demonstrated by peptide mapping of the antibody L chains. Radioautography of chymotryptic peptide maps of all three antibody L chains disclosed the presence of the same two unique variable region Cys-peptides which were not seen in the nonantibody L-chain pool. These two peptides appeared in different concentrations in C-I, C-II, and C-III L chains. Only half as much

radioactivity was present in these peptides in C-I and C-III, as compared with C-II. It is inferred from these data that approximately 50% of the L-chain molecules from C-I and C-III are strikingly similar to, if not identical with, C-II L-chain molecules.

Guinea pig antisera directed against individual antigenic determinants of C-II did not react in gel diffusion with non-antibody al b4 γG-globulin, nor with six other purified rabbit antibodies to Type VIII pneumococci. These antisera did cross-react with C-I and C-III to the extent of approximately 45%. Thus, three different types of experiments demonstrated the persistence of the same antibody species following three periods of immunization. These findings suggest that a single clone of cells, or the progeny thereof, persisted throughout several periods of immunization with pneumococcal vaccine. Similar observations have been made in rabbits repeatedly immunized with streptococcal vaccines or *p*-azobenzoate conjugates (Eichmann *et al.*, 1970; MacDonald *et al.*, 1969; MacDonald and Nisonoff, 1970).

Furthermore, the persistence for 29 months of related or identical idiotypic specificities in the antisalmonella antibodies of a single rabbit has been reported by Oudin and Michel (1969). These various observations are consistent with the view that a single cell or clone of cells may be capable of prolonged existence and repeated cycles of antibody production with interval phases during which production either diminishes or stops entirely. Alternatively, certain cells quiescent following initial exposure to antigen may upon subsequent stimulation undergo a single cycle of antibody production. Present evidence does not permit a definitive choice among these alternatives.

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