*AMINO ACID SEQUENCE RESTRICTION IN RABBIT ANTIBODY LIGHT CHAINS**

BY LEROY HOOD, HENRY LACKLAND, KLAUS EICHMAN, THOMAS J. KINDT,† DIETMAR G. BRAUN,‡ AND RICHARD M. KRAUSE

NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND; CITY OF HOPE MEDICAL CENTER, DUARTE, CALIFORNIA; AND THE ROCKEFELLER UNIVERSITY, NEW YORK CITY

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Abstract.—Light chains were obtained from IgG rabbit antibodies to the group-specific carbohydrates of groups A and C streptococci. An analysis of the amino acid alternatives which exist at the first three positions of the N-terminus in both light-chain preparations shows a marked restriction in amino acid sequence heterogeneity when compared with preimmune light chains. Both of these related, but immunologically distinct, antigenic determinants select the same uncommon subpopulation of rabbit light chains.

Introduction.—In occasional rabbits certain streptococcal cell-wall carbohydrates can stimulate the production of antibodies with uniform properties such as individual antigenic specificity and pronounced restriction of the electrophoretic mobility of the light chains.[1, 2] In this respect these antibodies bear a resemblance to the homogeneous myeloma proteins from which extensive amino acid sequence information has been obtained.[3, 4] This report is concerned with the initial studies to determine if the antibodies to streptococcal carbohydrates also possess sufficient molecular homogeneity to permit sequence analysis. The results of a semiquantitative Edman analysis on the first three positions of light-chain preparations from antibodies to group A and group C carbohydrates demonstrate in both cases a marked restriction in amino acid sequence heterogeneity when compared to normal light chains. In both instances the antibodies contained the same rare subpopulation of rabbit light chains.

Materials and Methods.—Immunization procedures: These procedures and the source of the rabbits have been previously described.[1, 2] Antisera were collected after the second immunization series of injections.[2]

Streptococcal cell walls and group-specific carbohydrates: Preparation of these materials has been previously described.[5, 6]

Serological, immunochemical, and electrophoretic methods: These methods have been previously described.[1, 2] Polyacrylamide gel disc electrophoresis was performed by the technique of Reisfield and Small.[7]

The method of quantitative precipitin analysis has now been modified so that the total protein in the immune precipitate, dissolved in sodium hydroxide, is measured in a Technicon autoanalyzer which has been adapted to perform the Lowry protein determination.[8]

Preparation of antibody light chains: Light chains were prepared from antibody by reduction, alkylation, and gel filtration.[9] Light chains were also prepared from the γ-globulin of serum of each rabbit collected prior to immunization. A homogeneous human kappa Bence-Jones protein, Hackney, was purified by DEAE ion-exchange chromatography and used to monitor the Edman procedure.

Determination of rabbit kappa and lambda light-chain ratios: The rabbit *b* allotype markers are found on kappa light chains.[10] The preimmune IgG and specific antibody of

rabbit R24-35 (allotype a2/b4) were labeled with ^{125}I and reacted against an anti-b4 antiserum.[11] The molecules bearing the b4 light chains (κ) were precipitated. The precipitate was washed and counted.

N-terminal analysis of light chains: The three-cycle Edman procedure was carried out on 8 to 12 mg of partially alkylated light chains.[12] The resulting PTH-amino acids were converted to free amino acids by hydrolysis with 6 *N* HCl for 24 hr at 150°C[13] and measured on the Beckman 120C amino acid analyzer. 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.[13]

Results.—Before proceeding to the amino acid analysis of the light chains of these antibodies, it is first necessary to describe the properties of the antibodies in these antisera in some detail and the rationale behind the isolation procedures. Prior studies have emphasized that the bulk of the total IgG in rabbit antisera to streptococcal carbohydrates may be either precipitating antibody or nonprecipitating antibody, or a combination of both.[2] For example, group C antiserum R24-35 contains 44 mg/ml of γ-globulin of which 90 per cent is precipitating antibody to the group C carbohydrate. Group A antiserum R22-85 contains 20 mg/ml of γ-globulin, of which 79 per cent is precipitating antibody to the group A carbohydrate.

It should be emphasized that each antiserum is specific and gives a negligible cross-reaction with the heterologous antigen when examined by quantitative precipitin tests.

Absorption studies of these antisera reveal that at least 95 per cent of the total γ-globulin can be specifically removed by cell walls containing homologous antigen, but not by heterologous cells walls. This is illustrated by the microzone electrophoretic patterns of unabsorbed and absorbed antisera depicted in Figure 1. The unabsorbed group A antiserum has two electrophoretically monodisperse γ-globulin components which are absorbed out by homologous, but not by heterologous, cell walls. The unabsorbed group C antiserum has one major slow component which is also specifically absorbed. In each case an analysis of the densitometric tracings of these electrophoretic patterns in conjunction with an analysis of the total serum proteins before and after absorption, by methods previously described,[1, 2] clearly ndicates that at least 90 per cent of the slow γ-globulin

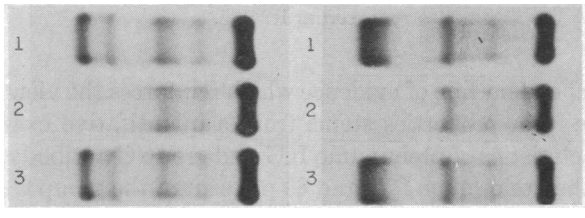

FIG. 1—Microzone electrophoretic patterns of antisera before and after absorption with homologous cell walls and heterologous group B cell walls.

Left panel: (*1*) top frame, group A antiserum R22-85; (*2*) middle frame, absorbed with group A cell walls; (*3*) bottom frame, absorbed with group B cell walls.

Right panel: (*1*) top frame, group C antiserum R24-35; (*2*) middle frame, absorbed with group C cells walls; (*3*) bottom frame, absorbed with group B cell walls.

component is absorbed with cell walls and is therefore antibody to the group-specific carbohydrate. For these reasons the slow component in each antiserum was isolated by preparative electrophoresis and was the antibody preparation employed in all subsequent analyses. Although such a procedure will not yield antibody as uniform as that obtained from specific immune precipitates, larger yields of material can be obtained for chemical analysis.

Depicted in Figure 2 are the electrophoretic patterns of the two antisera and their γ-globulin components isolated by preparative electrophoresis. Both isolated components are exclusively IgG when examined by immunoelectrophoresis. As illustrated in Figure 3, the light chains of group A antibody migrate as one major and several minor bands in 9.4 *M* urea polyacrylamide gel disc electrophoresis.[7] The light chains of group C antibody show greater heterogeneity, although the pattern is considerably more restricted than that observed for the light chains of preimmune γ-globulin.

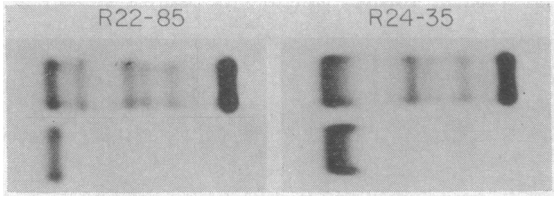

FIG. 2.—Microzone electrophoretic patterns of antisera and the monodisperse γ-globulin components isolated by preparative electrophoresis.

Left panel: group A rabbit antiserum R22-85.

Right panel: group C rabbit antiserum R24-35.

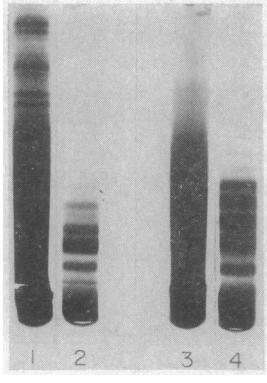

FIG. 3.—Polyacrylamide disc electrophoresis, pH 6.74 in 9.4 *M* urea of the reduced and alkylated γ-globulin components (illustrated in Fig. 2) which were isolated by the preparative electrophoresis. The direction of migration is from the bottom to the top. The heavy chains did not migrate beyond the interface of the upper and lower gel. Gel *1*, γ-globulin from preimmune serum of rabbit R22-85; gel *2*, isolated component from group A antiserum R22-85; gel *3*, γ-globulin from preimmune serum of rabbit R24-35; gel *4*, isolated component from group C antiserum R24-35.

Another independent line of evidence which reinforces the view that these antibodies have uniform properties stems from a quantitative examination of the allotypic characteristics of preimmune IgG and group C antibody, rabbit R24-35; 73 per cent of the preimmune IgG and 89 per cent of the group C antibody is precipitated with anti-b4 serum. Because this rabbit is typed as a homozygote at allotypic loci *a* and *b* and because the b4 marker is confined to the κ-chains,[10] it is inferred that the antibody possesses a larger proportion of kappa light chains than does the preimmune IgG. Although there was insufficient antibody from group A antiserum R22-85 for a similar study, it is likely, for reasons which are discussed later, that the same rare subpopulation of κ-chains was selected by both streptococcal cell-wall, group-specific carbohydrates.

N-terminal analysis: Table 1 gives the results of the Edman analysis for the first three residues on the groups A and C antibody light chains and the light chains from the rabbits' own preimmune IgG. Included, also, for comparison are the data on a homogeneous light-chain preparation, a Bence-Jones protein (Hackney). The duplicate determinations on this protein give an indication of the results that can be expected with essentially homogeneous light chains and some measure of the "noise" level present in this Edman procedure. The percentage of yield of each amino acid at each position has been calculated from the nanomoles of the specific amino acid and the total nanomoles of all the amino acids recovered at that particular sequence step. The percentage of recovery at each position has been calculated from the nanomoles of all amino acids at each sequence step and the nanomoles of dry weight protein used for the Edman procedure. For reasons that are not entirely clear, the recovery at any step can vary widely in repeated analyses of the same protein (see the results for Hackney steps 1 and 2 given in Table 1). However, the ratio of major amino acid residues obtained at each step is fairly constant. Repeated quantitative determination of the N-terminal residue of Bence-Jones protein samples agree

TABLE 1. *Amino acid residues at N-terminal sequence positions 1, 2, and 3 for the light chains of preimmune rabbit γ-globulin for antibodies to streptococcal carbohydrates and for the control Bence-Jones protein, Hackney.**

	N-Terminal Sequence Position					
	1		2		3	
Light chains	Yield† (%)	Recovery† (%)	Yield (%)	Recovery (%)	Yield (%)	Recovery (%)
Rabbit R22-85 pre-immune	Ala 54		Asp 34		Val 28	
	Asp 17		Val 28		Asp 19	
	Val 7		Gly 15		Ile 18	
	Ile 6		Glu 5		Met 5	
		70		18		39
Group A antibody	Ile 70		Val 81		Met 36	
	Ala 19		Gly 11		Val 22	
	Gly 9		Asp 5		Ile 14	
		37		31	Leu 10	24
Rabbit R24-35 pre-immune	Gly 43		Val 55		Val 33	
	Ala 31		Asp 10		Ile 15	
	Asp 10		Gly 10		Gly 14	
	Ile 7		Leu 9		Met 7	
		43		23		22
Group C antibody	Ile 70		Val 92		...‡	
	Ala 15		Gly 8		...	
	Gly 11		...		...	
		17		17		7
Bence-Jones protein (Hackney) analysis 1	Glu 100		Ile 89		Val 82	
			Gly 6		Gly 5	
		33		27		50
Bence-Jones protein (Hackney) analysis 2	Glu 96		Ile 67		Val 85	
	Gly 2		Gly 17		Gly 7	
	Asp 2		Glu 6		...	
		63		47		55

* Semiquantitative Edman procedure employed. See text for description of method.

† Calculation for percentage of yield and recovery are described in the text. Except at position 1, residue yields of 4% or less are not listed in the table.

‡ See discussion for omission of data at this position.

within ±2 per cent. At each successive step the reproducibility is less reliable and the noise level is greater. Based on the control samples presented in Table 1 and a larger series of controls that will be discussed elsewhere,[14] the following residue yields appear significant: any residue greater than 4 per cent at position 1, greater than 10 per cent at position 2, and greater than 15 per cent at position 3. Glycine may be an exception to these limits, due, in part, to the conversion of certain amino acids to glycine under the harsh conditions of acid hydrolysis, and possibly to the internal peptide bond cleavage at this labile residue during the Edman cyclization reaction. The results with the Bence-Jones protein show that there is less than 5 per cent trailing of the major amino acids from preceding steps.

The results with the preimmune light chains reflect the tremendous sequence heterogeneity of the normal light-chain population. At position 1 in the preimmune light chains of rabbit R22-85 there are four major amino acid alternatives with alanine predominating and isoleucine present at only the 6 per cent level. A similar picture is present in the next position where aspartic acid and valine are predominant, and at position 3 where valine, aspartic acid, and isoleucine are predominant. On the other hand, light chains of this rabbit's group A antibody show a striking decrease in heterogeneity at positions 1 and 2. At position 1 isoleucine has shifted from 6 to 70 per cent, and at position 2 valine has shifted from 28 to 81 per cent. Position 3 appears more heterogeneous than the other two, but the shift in methionine does represent a sevenfold increase in this residue over that observed in the preimmune light chains. In the case of the group C antibody light chains, the same shift to isoleucine and valine at positions 1 and 2, respectively, is present. A marked loss of yield at step 3 for these antibody light chains is consistent with the presence of either serine or threonine at this position because the hydroxamino acids are completely destroyed during acid hydrolysis. Complete use of the sample for acid hydrolysis precluded additional analysis.

Discussion.—Although the carbohydrate antigens of groups A and C streptococci consist of similar rhamnose moieties, in the case of group A the immunodominant determinants are β *N*-acetylglucosaminide residues, while in the case of group C they are *N*-acetylgalactosaminide residues. Rabbit antibodies to these related but distinct antigens have several properties which point to molecular uniformity. They possess individual antigenic specificity.[1] Their light chains are predominantly monodisperse when examined by disc electrophoresis. The allotypic data demonstrate a shift from 73 per cent κ-chains in preimmune IgG to 89 per cent κ-chains in the one antibody preparation tested. Finally, the amino terminal two residues for the light chains of both antibody preparations have a single major amino acid at each position. This restriction of amino acid sequence raises several questions which merit discussion.

The groups A and C antibody light-chain preparations studied here have predominantly N-terminal isoleucine, a relatively infrequent N-terminal residue in the light chains of preimmune γ-globulin. Since these antibodies possess different specificities, they must at some point have different amino acid sequences. Thus, at least two distinct sequences must exist in this relatively infrequent population of rabbit κ-chains with N-terminal isoleucine. Such infrequent sequences may represent two members of a kappa specificity (variable) region

subclass similar to those first described in man by Hood *et al.*[15] and by Smithies.[16] Three human kappa subclasses are defined by linked amino acid residues at certain positions and by sequence gaps (see ref. 15 for a discussion of these criteria). Thus, the N-terminal Ile-Val sequence which exists in a majority of the light chains in antibodies directed against both the group A and the group C antigens may be the first indication of a rare rabbit kappa specificity region subclass. In any event, both antigens have selected a similar infrequent subpopulation of rabbit κ-chains.

There are at least three examples in which a given antigenic determinant does not select a single light chain specificity region subclass. (1) The homogeneous human cold agglutinin antibodies (directed against the I determinant of red blood cells) which again are primarily the kappa type probably include at least two kappa specificity region subclasses.[17] (2) Rabbit anti-DNP light chains are qualitatively similar to normal rabbit light chains at the first six positions of the amino terminus, but quantitative differences are apparent.[20] No dramatic residue shifts comparable to the tenfold percent increase in isoleucine at the first position reported in the present study were observed. (3) In Hartley guinea pigs the DNP moiety selects an antibody light-chain population which is 95 per cent kappa type.[18] However, all the major guinea pig kappa specificity regions appear to be present in these light chains; indeed, the antibody light chains are virtually indistinguishable at their amino terminal four positions from nonimmune guinea pig light chains.[19] Thus, some antigenic determinants, such as those of streptococcal carbohydrates, appear to select a very restricted population of antibody light chains while in a number of instances others, such as DNP, do not. It seems probable that the DNP moiety is heterogeneous in that it is attached to the carrier protein at many different sites. This variability may give rise to a heterogeneous immune response with specific antibody drawn from different classes and subclasses. The streptococcal cell-wall antigens may possess greater regularity in chemical structure, a feature which may lead to greater uniformity in the immune response.

N-terminal amino acid analysis of light chains is now under way for streptococcal carbohydrate antibody which has been more highly purified than that employed here. If these antibodies do turn out to be homogeneous in primary amino acid sequence, comparative studies can be initiated on the structure of homogeneous antibodies to closely related but distinct antigenic determinants. Such comparative studies would be valuable in defining the nature of the antibody combining site. Furthermore, these studies, together with the sequence analysis of human and mouse myeloma proteins which possess specific antibody activity, will prove useful in probing the nature of the genetic machinery responsible for antibody sequence diversity.

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