INDUCTION OF RABBIT ANTIBODY WITH MOLECULAR UNIFORMITY AFTER IMMUNIZATION WITH GROUP C STREPTOCOCCI*

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Rabbit antibodies to streptococcal carbohydrates may possess several of the properties of the myeloma proteins which are indicative of uniformity. The antibodies are predominantly monodisperse on zone electrophoresis (1, 2) and possess individual antigenic specificity (3). The corresponding light chains show a restricted banding pattern on basic disc electrophoresis (3), and a marked restriction in the amino acid alternatives which are present at the first three positions of the N-terminus when compared to preimmune light chains (4).

Described in this report is an additional antibody to Group C streptococcal carbohydrate, isolated from a single rabbit, which appears, by a variety of criteria, to be as homogeneous as a myeloma protein. These data, and the unique allotypic properties of this antibody described elsewhere (5), indicate that immunization with streptococcal vaccines has stimulated the synthesis of a specific antibody with molecular uniformity. Furthermore, the N-terminal sequence of the light chains of this protein and two others previously reported (4) show a definite homology to the human Bence Jones proteins.

Materials and Methods

Immunization Procedures.—The source of rabbits, the preparation of streptococcal vaccines, and the immunization schedule have been previously described (6).

Streptococcal Group-Specific Carbohydrates.—The preparation of these materials has been previously described (7).

Serological and Immunochemical Methods.—These methods have been previously described (6).

The previous method of quantitative precipitin analysis (6) has now been modified so that the total protein in the immune precipitate, dissolved in sodium hydroxide, is measured in a

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Technicon autoanalyzer which has been adapted to perform the Lowry protein determination (8).

Electrophoretic Methods.—Microzone electrophoresis was performed on cellulose acetate membranes using a Beckman model R101 microzone cell as previously described (6).

Preparative electrophoresis was performed on 0.5% Seakem agarose in 0.05 M Veronal-HCl buffer, pH 8.6, as previously described (6). The protein was recovered from the gel fractions by centrifugation at RCF 39,900. The collapsed gel was washed three times with buffer. The protein was measured by the same procedure described above to measure the immune precipitates.

Polyacrylamide gel disc electrophoresis in 9.4 M urea was performed by the method of Reisfeld and Small (9). The gels were loaded with 200 μg of partially reduced and alkylated γ-globulin. Separation of light chains was obtained in 7.5 cm gels with a constant current of 2.5 ma/gel for 3.5 hr. For the separation of heavy chains, 4-cm gels were used with a constant current of 3 ma/tube for 6 hr. After staining with Coomassie blue and destaining, densitometric

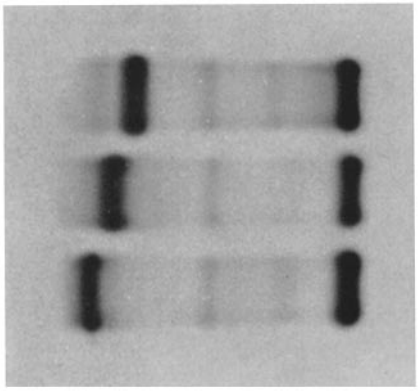

FIG. 1. Microzone electrophoretic patterns of three rabbit antisera to Group C streptococci Antiserum from rabbit R27-11, depicted at the top, was employed in the subsequent studies

tracings were obtained of the gel patterns at 650 mμ with a Gilford linear transport attached to a Gilford spectrophotometer.

Antisera to Rabbit Antibodies.—Antisera to rabbit antibody preparations were raised in goats by a previously described immunization schedule (3).

Preparation of Antibody Light Chains.—Light chains from antibody and normal rabbit γ-globulin were prepared by partial reduction, alkylation, and gel filtration according to the method of Fleischman et al. (10).

N-Terminal Analysis of the Light Chains.—The three cycle Edman procedure was carried out on 8–12 mg of partially alkylated light chains (11). The resulting phenylthiohydantoin (PTH) amino acids were converted to free amino acids by hydrolysis with 6 N HCl for 24 hr at 150°C (12) 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 (12).

RESULTS

Presented in Fig. 1 are the microzone electrophoretic patterns of three Group C antisera. Each has a sharp distinct component which consists of antibody

to the Group C carbohydrate. The monodisperse component may migrate in the fast, the intermediate, or the slow region of the γ-globulin pattern, suggesting that the net charge of these antibodies is unrelated to their specificity for the Group C antigen. The Group C antigen is a branched polymer of rhamnose

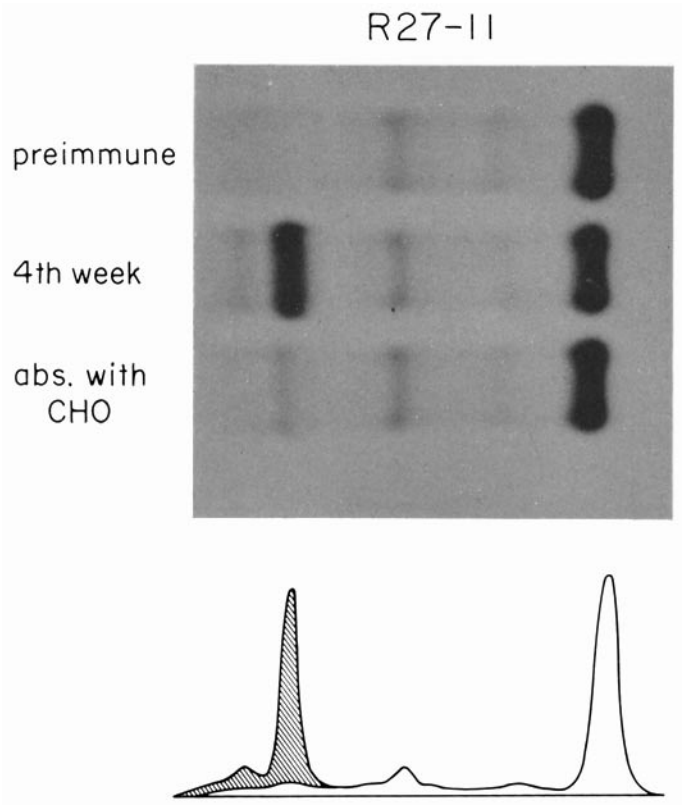

FIG. 2. Upper frame: microzone electrophoretic patterns. Upper pattern, preimmune serum of rabbit R27-11; middle pattern, antiserum collected after the 4th wk of immunization; lower pattern, antiserum after complete absorption with Group C carbohydrate. Lower frame: densitometric tracings of the microzone pattern of the antiserum before and after complete absorption with Group C carbohydrate. The shaded area represents the precipitating antibody removed from the antiserum at equivalence.

with terminal *N*-acetylgalactosaminide residues. Antigenic specificity is due to this immunodominant amino sugar (7).

The antibody in antiserum R27-11, depicted in the top frame of Fig. 1, will be described in detail in this report. This antiserum was collected after primary immunization.

In Fig. 2 are depicted the microzone electrophoretic patterns for the pre-

immune serum and the antiserum before and after complete absorption with Group C carbohydrate. The total γ-globulin in the unabsorbed antiserum was 45.6 mg/ml. The bulk of this, 36 mg/ml, was in the peak component, and the remainder in a broad shoulder fraction. Absorption of the antiserum with

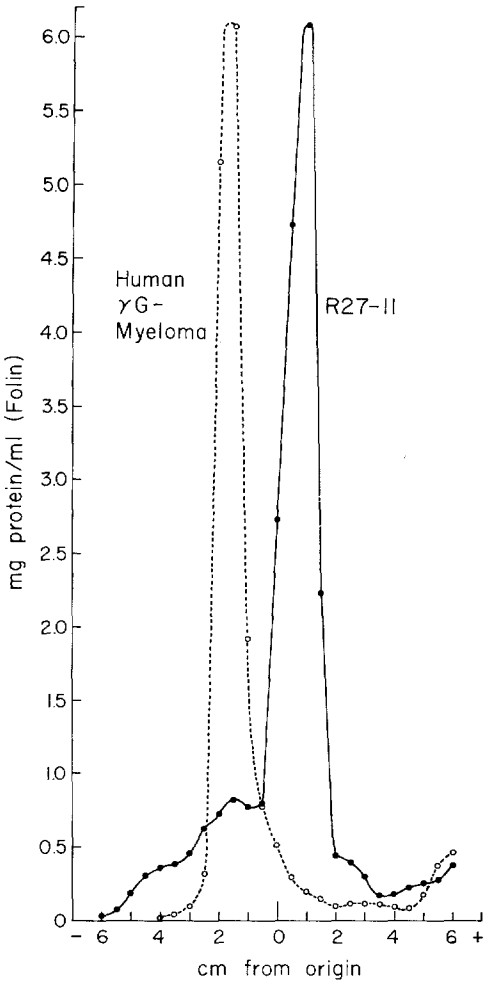

FIG. 3. Solid line: preparative agarose electrophoresis of antiserum R27-11. Only the protein curve for the γ-globulin region of the gel is shown. Dotted line: preparative agarose electrophoresis of a human myeloma protein.

Group C carbohydrate at equivalence removed 93% of the γ-globulin. As determined by quantitative precipitin tests, the antiserum contained 44.6 mg of antibody/ml. This amount of antibody was precipitated by 1.2 mg of Group C carbohydrate. This proportion of antigen to antibody is similar for most of

the other Group C antisera thus far examined. Calculated separately from the quantitative precipitin test and from the absorption data, between 93% and 98% of the total γ-globulin is antibody to the group-specific carbohydrate.

In view of these considerations, a specific immune precipitate was not employed to recover antibody from the peak component. Rather the peak component was isolated from the antiserum by preparative electrophoresis and employed without further purification as the antibody preparation. Agarose preparative electrophoresis of antiserum R27-11 is depicted in Fig. 3. Only the values for the protein eluted from the block in the region of γ-globulin are shown here. For comparison there is shown also the pattern for the serum of a patient with multiple myeloma. The distribution of the γ-globulin in the rabbit

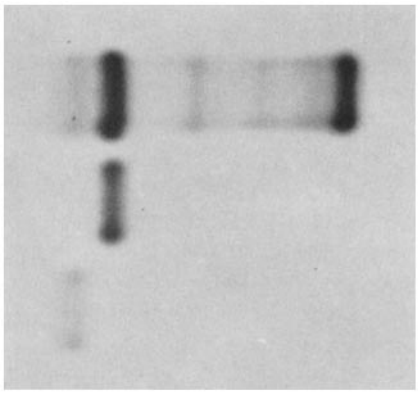

FIG. 4. Microzone electrophoretic patterns: upper frame, antiserum R27-11; middle frame, peak component; and lower frame, slow component.

antiserum is similar to that observed by microzone electrophoresis. Appropriate fractions of the block were pooled and concentrated to obtain the peak component and the slow component. The microzone electrophoretic distribution of each isolated component is depicted in Fig. 4.

In Fig. 5 are the 9.4 M urea disc electrophoretic patterns of the following partially reduced and alkylated proteins. These gels were specially prepared to resolve the light chains. Gel 1 contains normal rabbit γ-globulin. Gel 2 contains a Group C antibody with restricted heterogeneity. Gel 3 contains antibody R27-11. Gel 4 contains a human Bence Jones protein. The densitometric tracings of these gel patterns are depicted in Fig. 6. Normal rabbit light chains are usually distributed in 8–10 bands. The antibody with restricted heterogeneity yields light chains which resolved into four distinct light chain bands. Antibody R27-11 has one major band which contains at least 90% of the total light chain protein. This pattern is indistinguishable from that obtained with 40 μg of a human Bence Jones protein.

Depicted in Fig. 7 are the 9.4 M urea disc electrophoretic patterns of partially reduced and alkylated antibody R27-11 and a heterogeneous Group C antibody. These gels were constructed to resolve the heavy chains, and as a consequence the light chains have migrated beyond the gel. The densitometric tracings of these gels are shown in Fig. 8. Heavy chains of the heterogeneous antibody were resolved into 10 bands, whereas heavy chains of antibody R27-11 were confined to only 4 bands. Two of these bands appear predominant. Such a

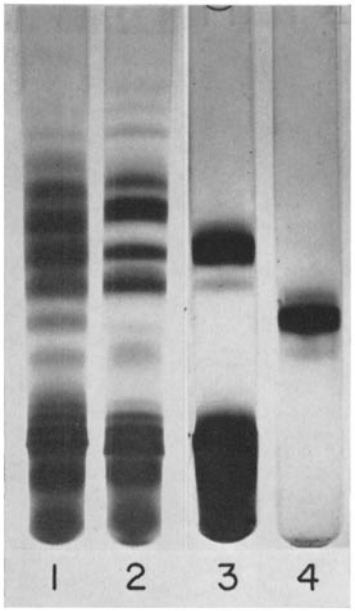

FIG. 5. Light chain patterns. Polyacrylamide gel disc electrophoresis, pH 6.74 in 9.4 M urea of reduced and alkylated γ-globulin preparations: gel 1, normal rabbit γ-globulin; gel 2, a Group C antibody with only partially reduced heterogeneity; gel 3, peak component of antiserum R27-11; gel 4, a human Bence Jones protein. The direction of migration is from the bottom to the top.

pattern may be seen with the heavy chains of myeloma protein (13). Allotypic studies which are reported elsewhere give additional indication that these heavy chains possess uniformity (5).

The case for uniformity of antibody R27-11 is further supported by the demonstration of individual antigenic specificity, a quality which certain antibodies share with myeloma proteins (3, 14, 15). A goat antiserum to the monodisperse antibody component of antiserum R27-11 was used in double diffusion experiments. Antibody R27-11 spurred over rabbit fraction II and over the polydisperse slow antibody component of antiserum R27-11. When the goat

antiserum was completely absorbed with fraction II, the reactions with fraction II and with the slow component were eliminated, whereas the reaction with antibody R27-11 remained.

Amino Acid Sequence of the Light Chain N-Terminus.—Tabulated in Table I

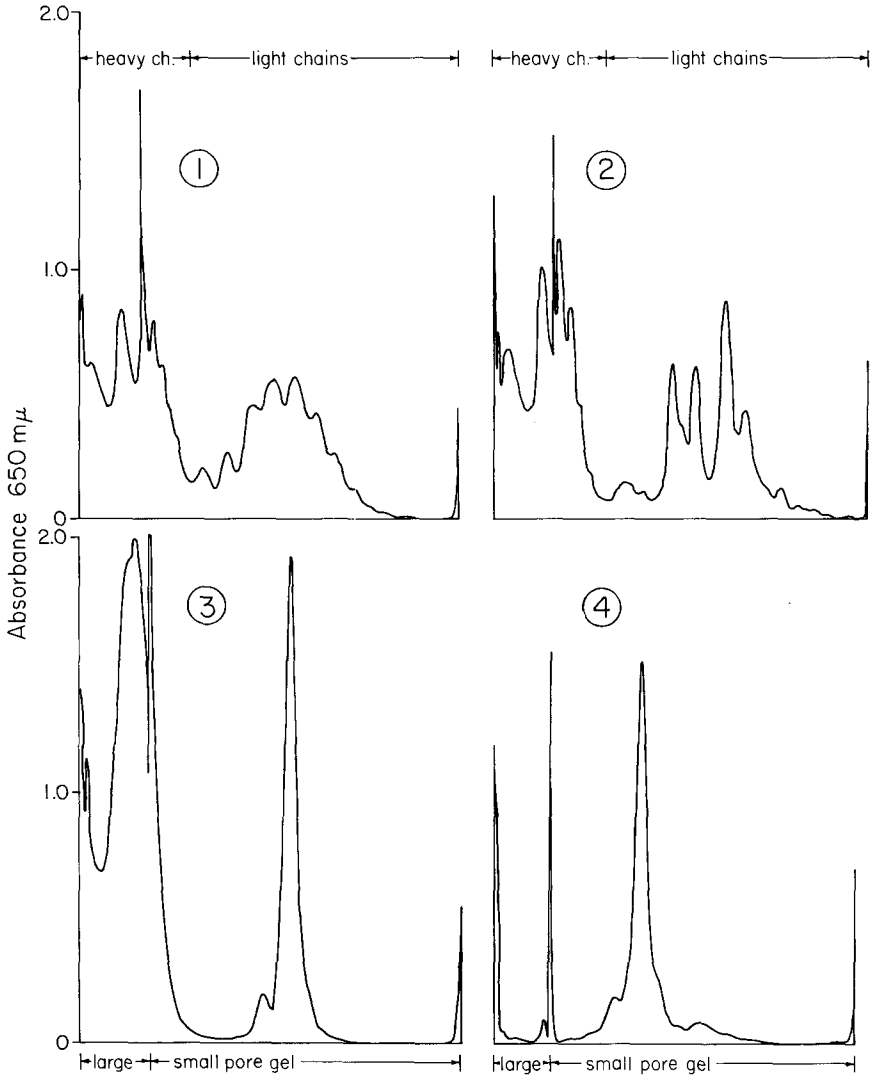

FIG. 6. Densitometric tracings of the disc electrophoretic patterns depicted in Fig. 5. Gel 1, normal rabbit γ-globulin; gel 2, a Group C antibody with only partially reduced heterogeneity; gel 3, peak component of antiserum R27–11; gel 4, a human Bence Jones protein. The direction of migration is from the left to the right.

are the results of the quantitative three cycle Edman analysis of the amino acid alternatives at the first three N-terminal positions of light chains of several different protein preparations. The amino acid data from antibody R27-11 was compared to that of the preimmune γ-globulin of the same rabbit, to that of a homogeneous human Bence Jones protein, and to that of the light chains from two previously described streptococcal antibodies which demonstrate

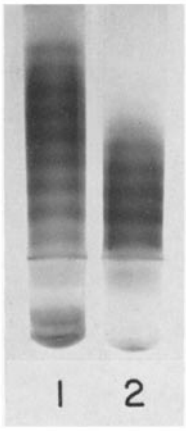

FIG. 7. Heavy chain patterns. Polyacrylamide disc electrophoresis, pH 6.74 in 9.4 M urea of reduced and alkylated γ-globulin preparations: gel 1, a Group C antibody with only partially reduced heterogeneity; gel 2, peak component of antiserum R27-11. The direction of migration is from the bottom to the top.

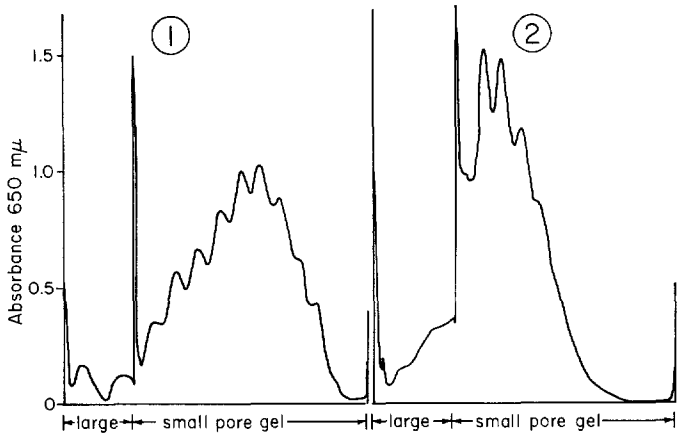

FIG. 8. Densitometric tracings of the disc electrophoretic patterns depicted in Fig. 7. Gel 1, a Group C antibody with only partially reduced heterogeneity; gel 2, peak component of antiserum R27-11. The direction of migration is from the left to the right.

TABLE I

Amino Acids at N-Terminal Sequence Positions 1, 2, and 3 for the Light Chains of Preimmune Rabbit γ-Globulin and for Antibodies to Group C Streptococcal Carbohydrate

Light chains	N-Terminal sequence position					
	1		2		3	
	Yield	Recovery	Yield	Recovery	Yield	Recovery
	%	%	%	%	%	%
Rabbit R27-11 preimmune γ-globulin	Ala 43		Val 31		Val 36	
	Asp 14		Asp 18		Asp 17	
	Ile 10		Glu 17		Glu 15	
	Leu 10		Tyr 8		Ile 11	
	Glu 7		Gly 7		Leu 11	
	Gly 5		Ala 5		Gly 10	
		46		26		8
Group C antibody	Ala 95		Asp 85		Val 92	
	Asp (2)		Val 4		Gly 8	
			Leu 4			
		48		26		10
Rabbit R22-85 preimmune γ-globulin	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		24
Rabbit R24-35 preimmune γ-globulin	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		Val 37	
	Ala 15		Gly 8		Met 26	
	Gly 11				Gly 13	
		17		17		7
Bence Jones protein (Hackney) analysis No. 2	Glu 96		Ile 67		Val 85	
	Gly 2		Gly 17		Gly 7	
	Asp 2		Glu 6			
		63		47		55

The quantitative Edman procedure was employed. The per cent 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 per cent recovery at each position has been calculated from the nanomoles of all amino acids at each sequence step and the dry weight of protein used for the Edman procedure. Except for position 1, residue yields of 4% or less are not listed in the table.

A portion of this data has been previously reported (4). It is included here to permit a comparison between antibody R27-11 and other streptococcal antibodies.

restricted heterogeneity. Calculations for per cent yield and per cent recovery are given in the table. The data for preimmune γ-globulin and antibody R27-11 were derived from the chromatograms of the hydrolyzed PTH-amino acids, depicted in Fig. 9.

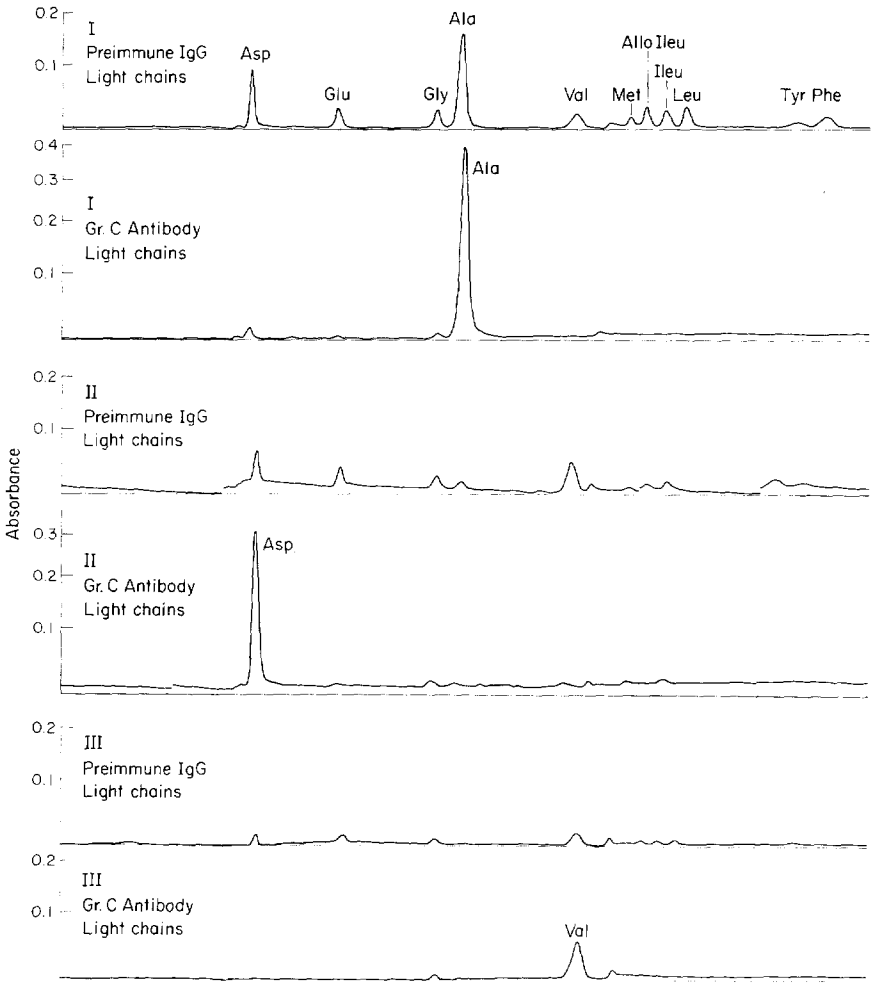

FIG. 9. Amino acid analysis chromatograms of the amino acids recovered from the first three N-terminal positions of the light chains of preimmune γ globulin and of the peak component of antiserum R27-11. I, first position; II, second position; III, third position.

The limits of the method employed for sequence analysis have been discussed elsewhere (4). It was concluded at that time that although the per cent recovery may differ markedly from step to step, the yield ratios reflect with reasonable accuracy the residue alternatives which exist at each position in a

given protein. Thus the light chains of the preimmune γ-globulin for normal rabbits exhibit tremendous heterogeneity with four or more amino acid alternatives at each of the first three positions. In contrast, the antibody light chains from rabbits R22-85 and R24-35 are markedly restricted in their heterogeneity. The dramatic shift from 6–7% to 70% isoleucine at the N-terminus of both antibodies suggests the possibility that 60 to 70% of the light chains belong to a highly restricted antibody population. Clearly, however, 15–19% of light chains from antibodies R22-85 and R24-35 have the common preimmune N-terminal residue, alanine, an indication that these are light chains with a sequence different from that of the predominant light chain population.

The light chains from antibody R27-11 appear to be comparable to a homogeneous human Bence Jones protein when examined by this Edman procedure. As shown in Table I, the yields of a single predominant amino acid at each of the first three N-terminal positions for antibody R27-11 resemble that for Bence Jones protein Hackney. No attempt to continue the sequence beyond position 3 was made by this method because of the rapidly decreasing yields.

DISCUSSION

In all of the previous reports which have been concerned with the immune response of the rabbit to streptococcal immunization, the evidence for molecular uniformity of antibodies to the carbohydrate antigens has been partial and indirect (1–3, 16, 17). Individual antigenic specificity of these antibodies is probably the one criterion, in the absence of sequence analysis, which is the most indicative of a uniformity similar to that observed for the myeloma proteins (14, 15). The predominantly monodisperse distribution of the light chains by disc electrophoresis is substantiating, though perhaps less rigorous, evidence. It remains to be determined, however, if antibodies with characteristics such as these, do, in fact, have a single primary amino acid sequence and are, therefore, homogeneous. N-terminal amino acid analysis of the light chains from antibody R27-11 reported here suggests that this may be the case.

The antibody to Group C carbohydrate from rabbit R27-11, described in detail here, possesses the following criteria which are indicative of uniformity: monodisperse distribution of the antibody by microzone and preparative zone electrophoresis, monodisperse distribution of light chains by disc electrophoresis, a markedly restricted banding pattern of the heavy chains on disc electrophoresis, and individual antigenic specificity. The special allotypic characteristics of this antibody which are described elsewhere (5) are further evidence for uniformity. For example, 99% of this antibody was precipitated by anti-b4 serum, a finding indicative of κ chains and the absence of λ chains, because the b4 marker is confined to κ chains.

The case for molecular uniformity of this antibody is considerably strengthened by the amino acid N-terminal analysis of the light chains. This revealed a

single amino acid at each of the first three positions in yields comparable to those seen for a homogeneous human Bence Jones protein. This represents a remarkable shift from the 4–6 major amino acid alternatives which were found in the three N-terminal positions of the light chains of the preimmune γ-globulin from the same rabbit.

Recovery of an antibody with this degree of uniformity from an antiserum is dependent upon several factors. Isolation is more readily achieved when antibody concentration in the antiserum is greater than 15 mg/ml. Furthermore, there must be a distinct predominant antibody component which is readily separated, by either preparative electrophoresis or some other means, from the remainder of the antibody and nonantibody γ-globulin in the antiserum. The situation is analogous to isolating a myeloma protein from human serum. For the purposes reported here, specific immune procedures were not required to isolate satisfactory antibody preparations, because 90% or greater of the protein isolated by electrophoresis is specific antibody. Subsequent purification by procedures which employ immune absorbents will be required, however, to prepare antibodies which meet more rigorous criteria for uniformity. This is clearly demonstrated by the allotypic studies on antibody R27-11 which made use of a form of specific immune adsorption in addition to preparative electrophoresis to achieve maximum uniformity (5).

Antibody R22-85, which was less uniform than antibody R27-11, is probably one which will possess greater uniformity when immune absorbents are used for preparation. In this antiserum, the major antibody component was not nearly as prominent as the one in antiserum R27-11, and thus the antibody preparation, recovered by preparative electrophoresis alone, was not devoid of other serum γ-globulin. As a consequence, the antibody preparation exhibits only restricted heterogeneity as judged by several criteria.

Antibody preparations, such as R22-85 and R24-35, may exhibit restricted heterogeneity rather than uniformity because they contain several antibody populations, each with a distinct amino acid sequence. It appears possible that immune absorbents can be used to resolve these individual populations and possibly render them as homogeneous as R27-11. This presents the exciting possibility that two or more homogeneous antibody preparations can be obtained from a single rabbit.

When the amino acid sequences of rabbit antibody b+ light chains are aligned against their human κ counterparts (18), a definite homology is suggested between the N-terminus of the human and the rabbit variable regions. Such an alignment is presented in Table II. Since a similar homology has been noted between the common region C-terminal peptides (19, 20), further support is gained for the hypothesis that b+ rabbit and human κ light chains descended from a common ancestoral κ gene in the variable as well as the common region. Furthermore, because of the addition of an extra N-terminal

alanine to some rabbit light chains (i.e., R27-11) and because of the deletion of the normal N-terminal residue in other rabbit light chains (i.e. R22-85), it is apparent why an N-terminal analysis of pooled rabbit light chains failed to reveal significant homology with human κ chains (19).

It is recognized that there may be less amino acid variability in this very N-terminal portion of the light chains of antibody R27-11 than is present in other parts of the sequence. It is, therefore, too early to predict that this antibody preparation will have a single amino acid sequence and is, without ambiguity, a homogeneous protein. Nevertheless, these data do indicate a molecular uniformity greater than that observed for antibodies to various determinants such as dinitrophenyl (DNP) which have been examined in a similar way (21, 22). For this reason, the uniform antibodies to streptococcal carbohydrates should be useful tools for probing the structure-function relationship of antigens and antibodies and the genetic mechanism responsible for antibody diversity.

TABLE II

Alignment of the N-Terminal Amino Acid Sequences to Show Homology between Human κ Light Chains and Rabbit Antibody Light Chains

Light chains	N-terminal position					Chain type
	0	1	2	3	4	
Man:						
Bence Jones protein*	—	Asp	Ile	Val	Met	κ
	—	Glu	(Val)	Gln	Val	κ
Rabbit:						
Group C ab						
R27-11	Ala	Asp	Val	Val‡	Met‡	κ (b+)
R24-35	—	—	Ile	Val	Met	κ (b+)
"	—	—	Ile	Val	Val	κ (b+)
Group A ab						
R22-85	—	—	Ile	Val	Met	κ (b+)
"	—	—	Ile	Val	Val	κ (b+)

* See Hood and Talmage (18). The valine at position 2 in human proteins is a rarely seen variant as indicated by the parenthesis. The two sequences for R24-35 and R22-85 light chains are justified by the obvious heterogeneity at position 3 in Table I.

‡ These residues at positions 3 and 4 have recently been determined by L. Hood and J. Ohms.

In rabbits the light chains of the b+ allotype are of the κ type.

SUMMARY

Antibodies with uniform properties may occur in rabbits after immunization with Group C streptococci. These precipitating antibodies possess specificity

for the group-specific carbohydrate. Not uncommonly, their concentration is between 20 and 40 mg/ml of antiserum. Evidence for molecular uniformity in the case of one of these antibodies, described in detail here, includes: individual antigenic specificity; monodisperse distribution of the light chains by alkaline urea polyacrylamide disc electrophoresis; and a single amino acid in each of the first three N-terminal positions of the light chains. When the amino acid sequence of rabbit antibody b+ light chains (κ type) are aligned against their human κ counterparts, a definite homology is observed between the N-terminus of the human and the rabbit variable region.

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