AN IDIOTYPIC CROSS-REACTION BETWEEN ALLOTYPE a3 AND ALLOTYPE a NEGATIVE RABBIT ANTIBODIES TO STREPTOCOCCAL CARBOHYDRATE*

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Several lines of evidence indicate that the idiotypic determinant of an Ig molecule is a reflection, at least in part, of the unique amino acid sequences of the binding site. For example, Wilson et al. (1) have described inhibition of idiotypic reactions between antibenzoate antibodies and their idiotypic antisera by various benzoate haptens. Idiotypic cross-reactions have been described among Waldenström's macroglobulins that have antibody activity for similar determinants. Williams et al. (2) described cross-reactions among a group of macroglobulins with cold agglutinin activity, and more recently, Kunkel et al. (3) reported a similar finding among macroglobulins with anti-γ-globulin activity. The latter study has been extended to show structural identity in certain hypervariable regions of the macroglobulins with similar idiotypes, while those with different idiotypes had differences in these regions (J. M. Kehoe and J. D. Capra, personal communication).

Idiotypic cross-reactions in selectively bred rabbits (4) and inbred mouse strains (5–8) point to the inheritance of idiotypic determinants and presumably antigen binding sites. The same idiotype was observed in the IgG and IgM antibodies isolated from the same rabbit (9) and idiotypic cross-reactions were also seen among human myeloma proteins of different classes (10, 11). These observations can be reconciled with the earlier finding of Todd (12) that the major immunoglobulin classes have the same H chain variable region allotypes (group a). Such findings suggest that synthesis of a given V_H region requires a gene or genes that carry information for the V_H allotypes as well as for the specific idiotypic markers.

A feature unique to rabbit antibodies is that both the group a allotypic markers and the specific idiotypic markers have antigenic components in the V_H region. This combination of markers provides the only known system to study the genes controlling synthesis of the V_H region. It would not be surprising if their close proximity to each other placed constraints on the possible combinations of idiotypic and allotypic markers. Earlier studies from this laboratory (4) suggested that such constraints may exist.

It was shown that rabbit IgG of the same allotype as the proband antibody inhibited reactions between the proband antibody and anti-idiotypic sera prepared in guinea pigs better than rabbit IgG of different allotypes.

In the example presented here, an obligatory association was not observed between group a allotypy and an idiotypic determinant detected by anti-idiotype sera prepared in rabbits. An idiotypic cross-reaction was observed between two different antibodies to the Group C carbohydrate isolated from the same rabbit antiserum. One antibody was allotype a3 while the other had no detectable group a allotype. Preliminary structural data have established no differences between the L chains of these two antibodies (13).

Materials and Methods

General.—Techniques for preparation of streptococcal vaccines, immunization of rabbits, and serological techniques have been previously described (14, 15).

Isolation of IgG, preparation of antiallotype serum, and radiolabeling techniques have also been previously described (16). Analytical electrofocusing in gels was carried out according to the method of Awdeh et al. (17).

Isolation of Antibodies.—Isolation of antibody from serum R3521 was initially carried out utilizing an immunoadsorbent column containing the hapten (p-aminophenyl-α-N-acetyl-galactosamine) coupled to Sepharose as previously described (15). Antibody was eluted as a single peak from this column by a solution of 0.5% N-acetylgalactosamine. By several criteria it appeared to be a uniform population of molecules (13).

Another immunoadsorbent, used for further resolution of antibodies, was prepared by coupling specially treated Group C carbohydrate to Sepharose. The carbohydrate was partially deacetylated and partially hydrolyzed by boiling under N_2 in 0.5 N $Ba(OH)_2$ for 20 min (18). The reaction mixture (5 ml containing 40 mg of carbohydrate) was desalted by passage through a column (2.5 × 26 cm) of Bio Gel P4 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated in H_2O. All excluded material was pooled and lyophilized. Extent of deacetylation of amino groups was monitored by the trinitrobenzene sulfonic acid test (19). The partially hydrolyzed carbohydrate was coupled to CNBr-activated Sepharose 2B by the method of Cuatrecasas (20). 5 g of CNBr were used to activate 80 ml of Sepharose. Uptake of carbohydrate was greater than 85%. The washed immunoadsorbent was reacetylated with acetic anhydride (18) and again washed. A column (1.2 × 20 cm) was poured at 4°C and equilibrated with 0.02 M citrate phosphate buffer, pH 6, containing 0.5 M NaCl. The sample was applied in this buffer, and a nonlinear pH gradient consisting of 250 ml of starting buffer and 9 ml of 1 M citric acid as a limiting buffer was used to develop the column. This specially prepared immunoadsorbent resolved the antibody recovered from serum R3521 into two populations of molecules.

Preparation of Idiotypic Antisera.—The anti-idiotype sera were prepared by injection of rabbits with isolated R3521 antibody. The injected rabbits were allotypically matched to rabbit R3521. The antibody was first injected subcutaneously in complete Freund's adjuvant, 5 mg a month for 3 mo. Intravenous injections were then given using purified antibody adsorbed to heat-killed pepsin-digested streptococci. 1 ml of the antibody-coated vaccine, containing an estimated 1 mg of antibody, was injected intravenously (i.v.) into rabbits at 3-wk intervals. Two rabbits produced precipitating antibody after the first i.v. injection. One of these died of anaphylactic shock upon receiving the second injection. The other was boosted and bled once a month over a 5 mo period and then exsanguinated.

Comparisons of idiotypic markers were carried out by radiobinding and inhibition of radiobinding assays as described previously (16). These methods were modified in that suspensions

of ethylchloroformate (ECF)[1] solidified antisera (21) were used in the assays in place of the liquid antiserum.

Recombination of H and L Chains.—Heavy and light chains were separated by the method of Fleischman et al. (22). The chains were concentrated to near dryness under nitrogen on an Amicon PM 10 membrane (Amicon Corp., Lexington, Mass.), suspended in 50 ml of 0.5 M acetate buffer, pH 4.5, concentrated to near dryness, and resuspended in 50 ml of water. Heavy chains used in inhibition studies were concentrated and stored in water at 4°C. The aqueous solution of the L chains was concentrated, taken up in 50 ml of saline, concentrated again, and stored in saline at 4°C.

H and L chains were recombined in equal molar amounts. A water solution containing approximately 5 mg/ml of H chains was mixed with a saline solution of L chains. The final protein concentration of the solution was approximately 500 μg/ml. This solution of H and L chains was allowed to stand at room temperature for 2 h before being used as an inhibitor of the radiobinding assay.

RESULTS

Isolation of the Antibodies.—As depicted in Fig. 1 A, an antibody component from serum R3521 was first isolated by affinity chromatography using an immunoadsorbent of *p*-aminophenyl-*N*-acetylgalactosamine bound to Sepharose. This antibody, designated Ab, was electrophoretically homogeneous by cellulose acetate electrophoresis. A single L chain band was seen on polyacrylamide disc gel electrophoresis in urea. The L chain had a single major sequence for 27 residues from the N terminus. Measurement of the group a allotype of this antibody, however, revealed that only 50% of the molecules had the a3 allotype; the remainder had no group a allotype. Compositional analyses of the H chain N-terminal peptides showed that at least two populations of H chains were present. It was concluded from these studies that Ab consisted of two different antibodies, each with similar or identical L chains, but with H chains different in their group a allotype and V_H sequence (13). Initial attempts to separate Ab into two or more components were not successful. The methods employed were agar block electrophoresis, preparative electrofocusing in sucrose over a range of two pH units, and several different immunoadsorbent columns, each utilizing several elution gradients.

Antibody preparation Ab was finally resolved into two components by elution with a very shallow acid pH gradient from a specially prepared immunoadsorbent column. The immunoadsorbent consisted of partially hydrolyzed Group C carbohydrate linked through its amino groups to Sepharose 2B. The material designated Ab in Fig. 1 A was concentrated, dialyzed, and applied to the second special column. Elution with an acid gradient, constructed using citrate phosphate buffer (Fig. 1 B), separated Ab into two components that eluted at pH 5.1 and 4.3, respectively. This is an indication of the close similarity in the affinity of the binding sites of these two antibodies.

The two antibody components, designated AbN and Ab3 in Fig. 1 B, were concentrated, dialyzed into phosphate buffer, and recycled through the special

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[1] *Abbreviations used in this paper:* Ab, antibody; ECF, ethylchloroformate.
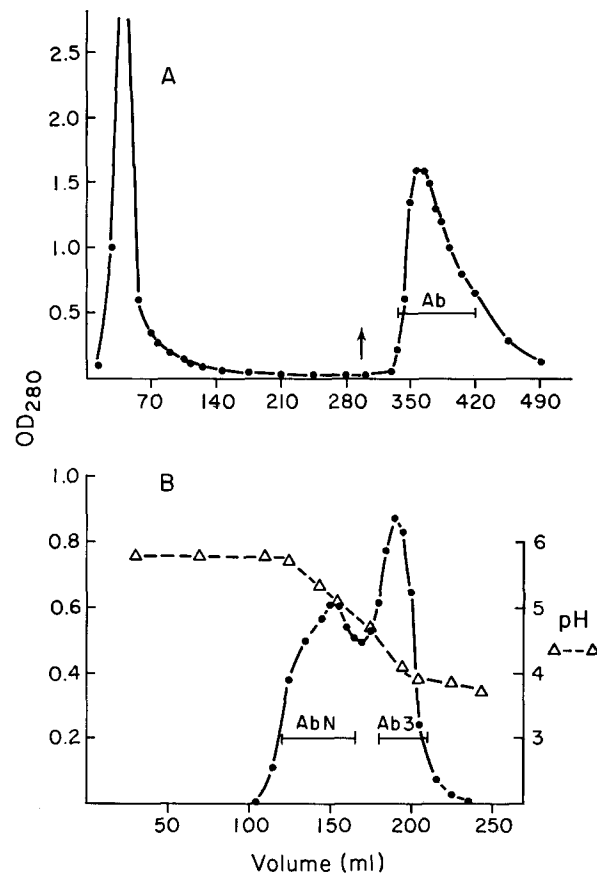
FIG. 1. (A) Adsorption and elution patterns of serum R3521 on an N-acetylgalactosamine Sepharose immunoadsorbent column. The arrow indicates the change to buffer containing 0.5% N-acetylgalactosamine. The fractions designated Ab were pooled, concentrated, dialyzed, and applied to the specially prepared immunoadsorbent column described in Fig. 1 B. (B) Resolution of the antibody into two components by use of the citrate-phosphate acid gradient on a column prepared by coupling partially hydrolyzed Group C carbohydrate to Sepharose 2B.

immunoadsorbent column. For each component, the pH required for elution was unchanged from that required for the elution in the initial separation.

After a second dialysis into phosphate buffer, the fractions were stored as concentrated solutions. Fig. 2 shows the results of electrophoretic analysis on cellulose acetate of the antiserum and the separated antibodies. While Ab appears to be one component, the separate antibodies AbN and Ab3 have slightly different mobilities in this electrophoretic system.

Electrofocusing in polyacrylamide gels provided a more detailed analysis of the differences between these antibody components. In Fig. 3 are seen the
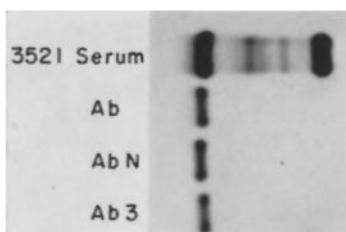
FIG. 2. Electrophoresis on cellulose acetate of R3521 antiserum, isolated Ab, and isolated antibody components. Serum albumin is to the right.
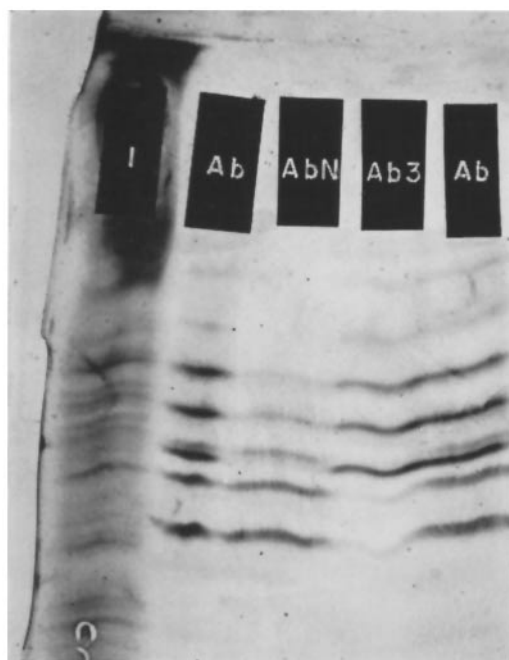

FIG. 3. Electrofocusing in polyacrylamide of R3521 Ab and antibody components. Sample designated 1 is whole serum from a nonimmunized rabbit.

banding patterns of Ab and the two antibody components isolated from it. Ab consists of six major bands, three of which are associated with AbN and three others associated with Ab3. Multiple bands are observed for the sample marked 1, which contains the total IgG fraction from nonimmune serum.

Allotypy of the Antibodies.—The a3 allotype of the [125I] antibody preparations was detected by binding to anti-a3 antiserum insolubilized with ethylchloroformate (ECF). Fig. 4 A gives the results obtained by addition of increasing amounts of insoluble a3 antiserum to a fixed amount of radiolabeled antibody. 43% of the unfractionated antibody, Ab, was bound to antiserum to allotype
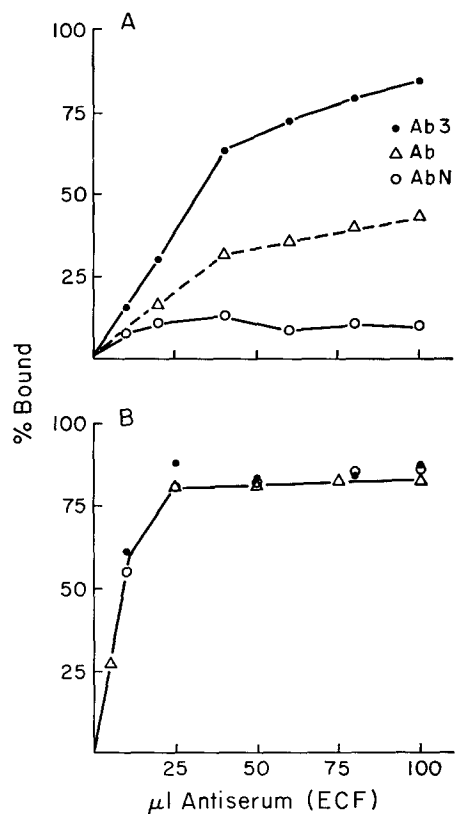
FIG. 4. (A) Binding of radiolabeled samples Ab, AbN, and Ab3 to ECF solidified anti-a3. Each tube contained 0.5 μg of sample and the amount of solid antiserum indicated on the abscissa. (B) Binding of radiolabeled samples Ab, AbN, and Ab3 to anti-idiotype serum prepared against R3521 Ab in an allotypically matched rabbit.

a3. In contrast, 85% of antibody component Ab3 was bound, while less than 10% of antibody component AbN reacted with the a3 antiserum. 90% of each antibody preparation was precipitated by antiserum to allotype b4 and by goat antiserum to rabbit IgG. It was concluded that the original antibody preparation had been resolved into two components: one possessing and the other lacking the a3 determinant. It is for this reason that the components were designated Ab3 and AbN; the former possessed the a3 determinant, the latter was negative for all group a determinants.

Idiotypy of the Antibodies.—Because the supply of the two isolated antibody components was too limited, anti-idiotypic sera were prepared against the unfractionated antibody Ab instead of the separate components. Anti-idiotype serum prepared in allotypically matched rabbits was used for the detection of idiotypy in Ab, and components Ab3 and AbN. Idiotypy was determined by

binding the radiolabeled antibodies to the anti-idiotype serum insolubilized with ECF. The results are shown in Fig. 4 B. It can be seen that nearly 85% of each preparation was bound to this anti-idiotype serum. This was also seen with an anti-idiotype serum prepared in a second rabbit.

Although these data suggest that the idiotypic determinants present in Ab3 and AbN are serologically identical, there are several questions that must be answered to refute several objections to this interpretation. First, is the reaction an idiotypic reaction or is it an allotypic reaction? Second, is this idiotypic cross-reaction due to shared determinants that are limited to only the L chains? This question is raised because the L chains of both components appear to be structurally identical (13). Third, are AbN and Ab3 reacting with the same antibodies in the anti-idiotypic antisera, or are there two populations of anti-idiotypic antibodies, one directed against each of these two antibody components? These questions were answered with a series of experiments to detect inhibition of binding to insolubilized anti-idiotypic serum by using various antibody preparations as inhibitors.

Listed in Table I are the inhibitors employed to inhibit the reaction between [^{125}I]Ab and ECF-insolubilized anti-idiotype antiserum. Both AbN and Ab3 gave nearly complete inhibition of this reaction. The F(ab')$_2$ portion of Ab rather than the Fc portion inhibits the idiotypic reaction. Neither isolated H or L chains inhibited the reaction, but a recombination of Ab H and L chains restored nearly all inhibitory capacity. The recombination product of Ab H chains and L chains from an IgG pool gave no inhibition. A similar result was

TABLE I

Specificity of Idiotypic Antiserum Against 3521 Ab as Detected by Inhibition of Binding of [^{125}I]3521 Ab With Various Inhibitors

Unlabeled inhibitor	Molar excess*	Inhibition‡
		%
3521Ab	10	95
3521Ab3	10	96
3521AbN	10	97
3521Ab F(ab')$_2$	12	91
3521Ab Fcγ	20	1
3521Ab H chain§	50	0
3521Ab L chain§	30	0
3521Ab H and L recombined	14	90
3521 preimmune IgG	10	0
3521 preimmune IgG	500	8

* The amount in excess of unlabeled inhibitor used to inhibit the reaction between ECF anti-3521 and 0.5 μg [^{125}I]3521 Ab.

‡ Percent binding in the tube with no inhibitor was considered 0% inhibition.

§ H and L chains were used as inhibitors both individually and after recombination with heterologous chains, heavy or light.

obtained with the recombination product of Ab L chains and H chains from an IgG pool. It required a 500-fold excess of preimmune IgG from rabbit R3521 to give 8% inhibition of the idiotypic binding reaction. It is concluded from these results that an idiotypic determinant was detected by the binding experiment depicted in Fig. 4 B, and that this idiotypic determinant requires a combination of the H and L chains of Ab.

The inhibition experiment depicted in Fig. 5 was done to determine if the antibodies in the anti-idiotypic serum react with the same determinant on both AbN and Ab3. As shown in Fig. 5 A and B, cold Ab, AbN, and Ab3 were all equally effective in the inhibition of the binding of [125I]AbN and [125I]Ab3 to anti-idiotypic serum. These data indicate that the same anti-idiotypic antibody in the idiotypic antiserum reacts with AbN and Ab3. This result was verified with the second anti-idiotype serum. The stoichiometry of the binding inhibi-
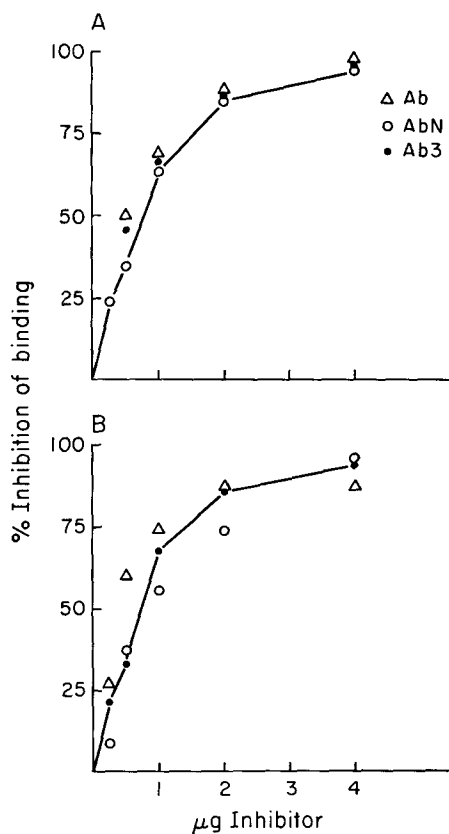

Fig. 5. (A) Inhibition of the binding of 0.5 μg radiolabeled AbN to ECF solidified anti-idiotype serum by unlabeled Ab, AbN, and Ab3. (B) The same inhibition experiment as in Fig. 5 A, but using radiolabeled Ab3.

tion tests precludes the possibility that this reciprocal inhibition by AbN and Ab3 is due to contamination of one component by another. It was concluded from these data that the idiotypic antisera to Ab are highly specific and detect a single major idiotypic determinant that is common to both AbN and Ab3.

The specific nature of the idiotypic relationship between AbN and Ab3 is further suggested by the absence of this determinant to any appreciable extent in the antisera of siblings. The total IgG fractions from the antisera of immunized siblings were used to inhibit the binding of radiolabeled Ab to an ECF preparation of anti-Ab. In one case a minor cross-reaction was observed. A 3,200-fold excess of an immune IgG preparation from one sibling was used to achieve 34% inhibition. The immune IgG's of all other siblings were even less effective as inhibitors.

DISCUSSION

Identical idiotypic determinants were detected by serological methods on two antibodies to Group C carbohydrate that were isolated from the same rabbit antiserum. Previous structural and serological studies established that the H chains of the two antibodies differed in group a allotypy, electrophoretic mobility, and N-terminal amino acid sequence while their L chains were similar if not identical (13). The procedure used for the isolation of these two antibodies took advantage of their somewhat different relative binding affinities under acidic conditions for a specially prepared immunoadsorbent column. One of the antibodies was allotype a3 while the other had no detectable group a determinant. The common idiotypic determinant was dependent on a combination of H and L chains.

The group a markers are absent in a small percentage of IgG molecules from a normal rabbit. The present case (R3521-AbN) represents the second report of homogeneous rabbit antibodies to streptococcal carbohydrate that lack the group a markers (23). The previously reported antibody was subsequently shown to carry the constant region allotype d12 (T. J. Kindt and C. W. Todd, unpublished observation). A different C_H allotype (A15) has been detected on group a negative IgG obtained from allotypically suppressed rabbits (24). The presence of IgG C_H allotypes on group a negative H chains implies linkage of group a negative V_H genes to the C_H genes, and therefore linkage to those genes coding for V_H regions carrying the a allotypes. The two types of V_H regions, a positive and a negative, might represent IgG subgroups in the variable region.

The a3 allotypic determinant on the H chain of one antibody and the absence of a group a allotype on the H chain of the other must be the result of certain amino acid substitutions in the V_H region. While the number and positions of the amino acid substitutions involved in the group a allotypic determinant have still to be determined (25, 26),[2] it is likely that the specificity of each determinant requires multiple replacements. Serologically identical idiotypic determi-

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[2] E. Haber and A. D. Strosberg. Unpublished data.

nants on the two antibodies were observed despite the allotype-related differences that must exist in their respective V_H region sequences. In this instance, at least, such a finding argues against obligatory associations between the group a allotypic markers and specific idiotypes as detected by antisera produced in allotypically matched rabbits.

The occurrence of the same idiotype on two antibodies with different V_H region allotypes has immunogenetic implications. The idea that an immunoglobulin chain is the product of two or more genes has been widely discussed (27, 28). The premise that a large number of V_H genes may in some way interact with a relatively small number of C_H genes simplifies but does not solve the problem of antibody diversity. How an individual maintains and utilizes a given set of V_H and V_L genes is still a mystery.

There are two or possibly three categories of information that are necessary for the synthesis of the V_H region of a given rabbit antibody. First, information is needed for those portions of the V_H region that are constant in an individual species. The genetic polymorphism that occurs in the V_H region of the rabbit (group a allotypy) may require information in an additional category, or alternatively this information may fall into category one. The latter alternative is favored because it simplifies the situation. The second category will then contain information required for synthesis of the binding site sequence. The recently developed concept of hypervariable regions would dictate placement of the residues directly involved in antigen binding at fixed positions in the antibody H chain (29). The heavy chain contribution to the binding site appears to consist of three or more peptides, each consisting of approximately five residues and each placed at critical positions in the V_H region of the chain. How then are the informational segments needed for V_H synthesis genetically encoded? Because group a allotypes and idiotypes have antigenic components in the V_H region their genetic relationship is directly pertinent to this question.

Recent studies on the inheritance of idiotypic determinants in rabbits and in inbred mice suggest that the information for antigen binding sites is inherited (4–8). A possible linkage relationship between the group a allotypic and the idiotypic determinants was suggested by previous studies from this laboratory. The discrepancy between this previous finding and the absence of an idiotypic-allotypic relationship reported here is presumably the result of using different idiotypic antisera. In the previous studies inhibition of precipitin reactions between heterologous idiotypic antisera prepared in guinea pigs and proband antibodies showed that IgG of the same group a allotype as the proband was a better inhibitor of the reaction than IgG's of different group a allotypes. It is conceivable that these heterologous idiotypic antisera recognized certain elements of the allotypic marker as well as the idiotypic marker. For this reason it was suggested that molecules with the same group a allotypic determinant were better inhibitors of heterologous idiotypic reactions by virtue of interaction of allotypic and individually specific (idiotypic) determinants (4). This

explanation is consistent with the results of the present study which utilized homologous idiotypic antisera prepared in allotypically matched rabbits. In this case, the group a allotypic determinant had no influence on the idiotypic reaction, presumably because the homologous idiotypic antisera did not recognize any elements of the allotypic determinant.

Two simple alternative mechanisms can be postulated for the occurrence of the same idiotype on two different H chains, one with a group a allotypic marker and one without a group a allotypic marker. First, two copies of the V_H (or H) genes for this binding site might be maintained, one that possesses a group a marker and one that does not. Second, a gene that codes for the binding site, but is separate from the V_H gene that codes for the constant portion of the V_H region, may insert the information for the binding sites and therefore the idiotypic determinant. The former possibility would involve a large number of germ line genes carrying duplicate information and would involve obligatory associations between group a allotypes and idiotypes. Such a situation is contrary to the findings reported here. An explanation for the second possibility might involve episomal genes as suggested earlier by Wu and Kabat (29).

If the second alternative is true and separate V_H genes can be maintained, shared idiotypy among allotypically different molecules should be observed. There are as yet insufficient observations to determine which, if either, alternative mechanism is the most likely. It is pertinent to note that Kehoe and Capra (unpublished studies) found nearly identical amino acid sequences in certain hypervariable regions of several antibodies with idiotypic cross-reactivity although these antibodies had differences in portions of the V_H region other than the hypervariable regions. The streptococcal antibodies with idiotypic cross-reactions reported here may represent an analogous situation, but additional sequence data are required to firmly establish the analogy.

A complete analysis of the number and nature of V_H genes and their interactions will require extensive breeding studies as well as amino acid sequence data on molecules with cross-reactive idiotypic determinants. For example, in the present case, it is impossible to determine whether the two different H chains were of the same or of different parental origin, because rabbit R3521 was homozygous with respect to the C_H allotypes. Further studies on idiotypic cross-reactions among antibodies from animals that are heterozygous with respect to C_H as well as V_H allotypes may help delineate the relationships among V_H genes.

SUMMARY

Two antibodies to Group C streptococcal carbohydrate isolated from an individual rabbit had similar relative binding affinities for a Group C immunoadsorbent column. Their light chains were similar, if not identical, as were the constant regions of their heavy chains. Differences in the variable regions of the H chains of the two antibodies were detected by chemical analysis. The two

antibodies had serologically identical idiotypic determinants although one antibody possessed the a3 allotype and the other had no detectable group a marker. The occurrence of such antibodies indicates the absence of obligatory associations between group a allotypes and idiotypic specificities, despite the fact that both determinants have antigenic components in the V_H region of the H chain.

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