DETECTION OF IDIOTYPIC CROSS-REACTIONS AMONG STREPTOCOCCAL ANTISERA FROM RELATED RABBITS*

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Recent genetic studies on the immune response suggest that idiotypic markers of antibodies are inherited. Idiotypic cross-reactions were detected among the antibodies to the Group C carbohydrate from related rabbits while these same idiotypic specificities were not seen in unrelated rabbits (1). Genetic studies with inbred mice immunized with various bacterial and synthetic antigens indicate that idiotypic markers are transmitted from parents to offspring (2–5).

Various lines of evidence suggest that amino acid substitutions associated with the antigen-binding site are part of the idiotypic determinant(s) of an antibody (6). For example, idiotypic cross-reactions are seen among certain groups of human myeloma proteins which have antibody activity for the same antigenic determinant (7, 8). Genetic studies utilizing idiotypic determinants may, therefore, be helpful in determining the mechanism of inheritance of variable regions associated with antigen binding sites.

For several reasons the rabbit is a useful animal for the study of inheritance patterns of antibody variable regions. A major advantage is that both the idiotypic markers and the group a allotypic markers are present in the variable region of the heavy chain (9). Furthermore, analytical methods are available for the detection of allotypic markers identified on both the heavy and light chains. Thus, both idiotypic and allotypic markers can be employed for genetic studies on the rabbit antibody variable region. Despite these advantages, there have been several obstacles which hampered the use of rabbit idiotypy as a genetic marker. A major experimental difficulty stems from large differences in the level of expression of antibody with cross-reacting idiotypes among related rabbits. Another deterrent to the use of rabbit idiotypes of streptococcal antibodies in genetic studies is the occurrence of IgG and IgM anti-IgG's in the antisera of rabbits immunized with streptococcal vaccines (10). As will be shown here, these anti-IgG's can mask the presence of antibodies

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with idiotypic cross-specificity in certain test systems such as quantitative precipitin inhibition. Use of alternative methods can circumvent this effect.

In an earlier report (1), idiotypic cross-reactions were detected among the Group C antibodies from rabbits inbred for several generations. The idiotypic antisera to detect this idiotypic cross-reaction had been prepared in guinea pigs and absorbed with pooled rabbit IgG. Such idiotypic antisera probably detect a broader specificity in the variable region than do the idiotypic antisera prepared in the same species. The present study demonstrates that idiotypic cross-reactions detected by idiotypic antisera prepared in allotypically matched rabbits are commonly seen among streptococcal Group C antibodies produced in related rabbits, but are rarely detected among antibodies from unrelated rabbits.

Materials and Methods

General.—Techniques for preparation of streptococcal vaccines, immunization of rabbits, cellulose acetate electrophoresis, and serologic techniques for antibody determinations have been previously described (11, 12). Isolation of IgG, preparation of antiallotype sera, and radiolabeling techniques have also been previously described (13). Antibodies were isolated by immunoabsorbent columns and further purified by agarose block electrophoresis. Antibody preparations were tested for homogeneity as previously described (11). Heavy and light chains were separated by the method of Fleischman et al. (14), and recombinations of heavy and light chains carried out as described by Kindt et al. (15).

Preparation of Idiotypic Antisera.—Idiotypic antisera were prepared against isolated proband antibodies. A 1 ml saline suspension of homogeneous antibodies, cross-linked by the gluteraldehyde method described by Daughtery et al. (16), and containing approximately 1 mg of protein, was homogenized with 1 ml of complete Freund's adjuvant and injected subscapularly at 3-wk intervals into allotypically matched rabbits. 3 wk after the third injection, the rabbits were given an intravenous (i.v.) injection of 1 ml of homogenized gluteraldehyde cross-linked antibodies. The rabbits were bled at 2-wk intervals throughout the immunization period. Solidification of antisera was carried out by the ethylchloroformate (ECF)[1] procedure of Avrameas and Ternynck (17).

Detection of Idiotypes and Cross-Idiotypic Specificities.—Idiotypes were determined by radioprecipitin analysis and radioprecipitin inhibition (RPI) tests, radiobinding and radiobinding inhibition (RBI) analysis, and hemagglutination and hemagglutination inhibition (HI) tests.

Radioprecipitin Analysis.—Precipitin analyses for detection of idiotypic determinants were carried out on the radiolabeled proband antibodies as previously described by Kindt et al. (13) using 5μg of radiolabeled antibody and increasing amounts of the appropriate antiserum.

Radioprecipitin inhibition tests were carried out as previously described. In the inhibition studies described here, whole antistreptococcal sera were used as inhibitors. These were added to a predetermined amount of anti-idiotype sera which was chosen to achieve antigen excess with respect to ^{125}I-labeled proband antibody. The inhibitor and idiotypic antisera were mixed and incubated for 2 h at room temperature before the addition of radiolabeled proband antibody. After this addition, the tubes were again mixed and incubated 2 h at room tempera-

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; ECF, ethylchloroformate; HI, hemagglutination inhibition; RBI, radiobinding inhibition; RPI, radioprecipition inhibition.

ture and then overnight at 4°C. Centrifugation, radiocounting, and calculations were carried out as previously described (13).

Radiobinding Analysis.—Binding analyses were carried out in similar fashion to the precipitin analysis described above except that ECF solidified antisera were used. In these assays, 0.5 μg of ^{125}I-labeled antibody and various dilutions of the solid idiotypic antiserum suspension were used to construct binding curves. The labeled antibodies were added first and the tubes were mixed immediately upon addition of the solid idiotypic antisera. The tubes were then mixed on a rotary shaker for 3 h at room temperature and allowed to sit overnight at 4°C.

RBI tests for the detection of idiotypic markers were carried out using whole streptococcal antisera, isolated IgG, and antibody fractions as inhibitors. A point in antigen excess with respect to the proband antibody was chosen. The inhibitor was added to solid antisera with mixing and the radiolabeled proband added, and the tubes were mixed again. The tubes were shaken and incubated as in the direct binding procedure. Centrifugation and calculations were similar to those performed for the precipitation tests.

Hemagglutination and Hemagglutination Inhibition Assays for Detection of Idiotypic Cross-Reactions.—Hemagglutination reactions utilized rabbit type F red blood cells (RBC) coated with antibody preparations by the chromic chloride method of Gold and Fudenberg (18). The reactions were carried out in plastic microtiter plates (Linbro, New Haven, Conn.) Antisera titers were determined by addition of 50 μl of serial dilutions of antisera to the wells followed by addition of 50 μl of a 2% suspension of coated RBC's in hemagglutination buffer (Difco Laboratories, Detroit, Mich.) containing 1% bovine serum albumin (BSA). The plates were covered and stored at 4°C. They were read after 2 h and again after incubation overnight. Titers were recorded as the reciprocal of the dilution in the last well to show visible hemagglutination. Each coated RBC preparation was checked with several antiallotype sera for the presence of appropriate allotypic markers and absence of nonspecific agglutination. Each antiserum was tested for agglutination with uncoated rabbit RBC.

HI reactions to detect idiotypic cross-reactions utilized the dilution of antisera present in the well before that containing the last agglutinated cell suspension. 50 μl amounts of inhibitors were serially diluted and 25 μl of the appropriate dilution of antisera was added to each well. After mixing, 25 μl of a 4% suspension of coated RBC's was added. Plates were incubated and scored as before.

When whole streptococcal antisera were used as inhibitors, it was necessary to check for agglutination of the coated RBC's caused by anti-IgG in the antisera (10). Preliminary experiments indicated that few, if any, streptococcal antisera would agglutinate cells coated with homogeneous antibodies when the test sera were diluted 1:10. For this reason, an initial 1:10 dilution of all antisera was used in the HI test to detect idiotypic cross-reaction.

HI experiments were scored according to the concentration of the sample in micrograms per milliliter of IgG in the last well which inhibited agglutination (that is, the last well where visible settling of the coated RBC's occurred). The percentage of the total immune IgG in the test antisera which had idiotypic cross-specificity was calculated by comparison of the IgG concentration of the test sample to that of the homologous inhibitor sample that gave similar inhibition. In a typical experiment, a solution of 1 μg/ml of the antibody used to coat the RBC's would be the last dilution to give clear inhibition. If a test serum dilution containing 500 μg/ml of IgG from a related rabbit gave similar inhibition, it could then be calculated that 1 in 500 or 0.2% of the IgG molecules present in the test sample had the cross-idiotypic specificity. The limit of detection in the assay, used in this way was about 0.1% (1 μg/ml in total of 1 mg/ml IgG) cross-reacting antibody. This limit is set because of the aforementioned 1:10 dilution of the antisera which is done to circumvent the problem of nonspecific agglutination caused by the anti-IgG present in the antistreptococcal sera tested.

Measurement of Anti-IgG Levels.—Anti-IgG levels were measured by methods described by Bokisch et al. (10). 19S anti-IgG was measured by a hemagglutination assay using rabbit F

RBC's coated with anti-F and 7S anti-IgG was determined by a heterologous coprecipitation method. The 19S and 7S anti-IgGs were separated on Sephadex G-200 (100 × 2.5 cm). The IgG and IgM concentrations in the individual fractions were measured by the radial diffusion method of Mancini et al. (19).

RESULTS

Prior studies on familial idiotypic cross-reactions employed antibodies to Group C carbohydrate which were obtained from members of a closed rabbit colony which had been immunized with Group C streptococci (1). Since the completion of that work, this colony has been continually inbred during the last 2 yr. The Group C antisera from all the rabbits used in the previous study, as well as those born in the interval, served as the basis for this work.

Rabbit 3412 of the F_2 generation of this inbred family was selected as one of the probands because its antiserum contained a major antibody component with restricted heterogeneity. This antibody was readily isolated by preparative agarose electrophoresis. The isolated antibody was homogeneous by various criteria including a single amino acid sequence for the first 20 N-terminal residues of the light chain. The rabbit was allotype a2,3/b4 and the purified antibody was a2/b4.

The other proband rabbit was 2690, and antiserum from this rabbit also contained a predominant monodisperse antibody component. The antibody was isolated by agarose block electrophoresis, and immunoadsorbent chromatography. This antibody has been the subject of extensive structural studies which point to molecular uniformity (20).

Several methods were employed to detect idiotypic cross-reactions. With some methods the results were influenced by the presence of anti-IgG's in the streptococcal antisera. HI was finally selected for the bulk of the work reported for reasons discussed below. Before results with this method are presented, however, efforts to detect idiotypic-cross-reactions with the RPI test will be described, because in this case, it was shown that anti-IgG's in the streptococcal antisera obscured the detection of idiotypic cross-reactions.

Influence of Anti-IgG's on Detection of Idiotypic Cross-Reactions.—In the initial studies on the search for cross-reactions to the idiotype of antibody 3412, the RPI test was selected because the anti-3412 idiotypic antiserum precipitated 70% of the isolated 3412 antibody. It was anticipated that addition of antisera from other rabbits containing cross-reacting antibodies would inhibit the precipitation of [^{125}I] 3412 antibody in the RPI test, whereas no inhibition would be observed with antisera in which such antibodies were absent. What, in fact, occurred was that the Group C antisera from almost all rabbits of the pedigree enhanced the amount of precipitation over that observed in the control.

It was postulated that 19S and 7S anti-IgG's which occur in virtually all antistreptococcal antisera might be the cause of the enhanced precipitation

observed in the RPI test. To examine this possibility, the RPI test was set up, as usual, with [^{125}I] 3412 antibody and its anti-idiotype. A standard inhibition curve was obtained by addition of increasing amounts of purified unlabeled 3412 antibody. It was shown that the purified antibody contained no anti-IgG's. The inhibition test was repeated, but to each tube was added 12.5 μl of a Group C antiserum from a proband rabbit. This antiserum had a hemagglutination titer of 1:2,600, an indication of a high concentration of 19S anti-IgG. This Group C antiserum also contained 13.2 mg/ml of 7S anti-IgG. The amount of [^{125}I] 3412 Ab precipitated was enhanced twofold in the presence of the Group C antiserum from this immunized sibling. Addition of as much as 8 μg of unlabeled 3412 Group C antibody, an amount which gives nearly 100% inhibition in the control test, did not alter this enhancement. Because of this masking effect, a Group C antiserum, such as the one tested above, could contain as much as 1 mg/ml of an antibody with the 3412 idiotype and not be detected by this RPI test.

The experiment depicted in Fig. 1 was done to determine if either the 19S or 7S anti-IgG was primarily responsible for this enhancement of precipitation. IgG and IgM preparations were isolated from an unrelated Group C antiserum and added to the RPI test containing [^{125}I] 3412 streptococcal antibody and anti-3412. While somewhat greater enhancement was seen with the whole

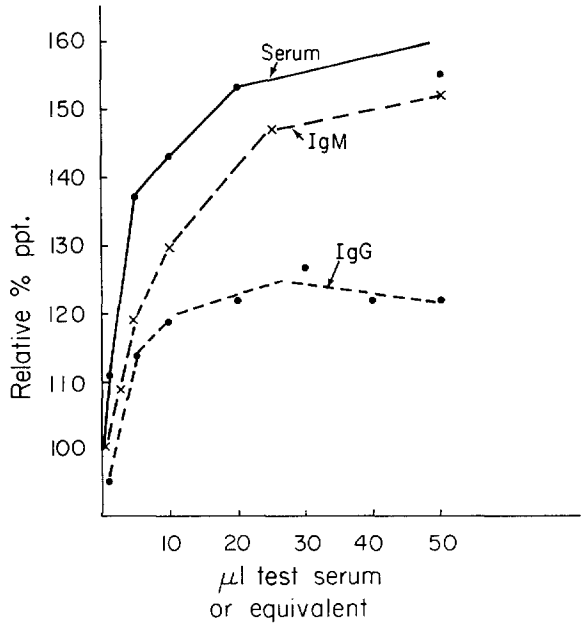

FIG. 1. Enhancement of radioprecipitation of 3412 [^{125}I] Ab with anti-3412 by a test antiserum containing 19S and 7S anti-IgG's (●—●) and IgM (×--×) and IgG (●--●) fractions from this antisera.

serum, the IgM fraction utilized at the original serum concentration was nearly as effective. The IgG fraction was the least effective even though the concentration of the 7S anti-IgG was considerably greater than the 19S. A homogeneous anti-IgG of the IgG class isolated from the serum of rabbit 3387 and added to the RPI test gave the lower level of enhancement similar to that seen with the IgG fraction.

Detection of Idiotypic Cross-Reactions by the RBI Test.—Because the anti-IgG's in streptococcal antisera adversely influence the results with the RPI test for detecting idiotypic cross-reactions, the RBI test was used to determine if this interference could be circumvented. The experiment depicted in Fig. 2 indicated that antisera containing anti-IgG's did not mask the inhibition in the RBI test. The inhibition curve, obtained by addition of increasing amounts

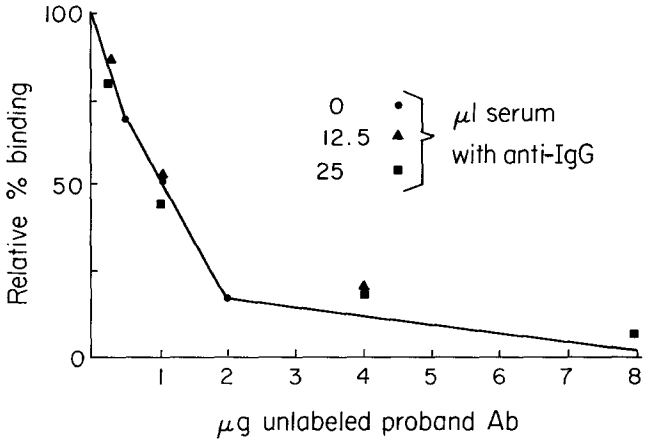

FIG. 2. Inhibition of binding of [^{125}I] 3412 Ab to ECF anti-3412 in the presence of buffer (●), 12.5 μl (▲), and 25 μl (■) of serum with IgM and IgG anti-IgG.

of unlabeled 3412 antibody, is not altered in the presence of streptococcal antisera containing anti-IgG's. The test antiserum used in this experiment was the same as the one used in the RPI experiment described above.

Group C antisera from rabbits related to 3412 were tested by the RBI test for idiotypic cross-reactions with 3412 antibody. 50 antisera from four generations of the family were tested and not one cross-reaction was detected. Such a result suggests that the idiotype of this antibody is rarely expressed even among these relatively inbred rabbits.

The detection of idiotypic cross-reactions is enhanced if the test system employs an immunoglobulin other than the one against which the idiotypic antiserum was prepared. The use of idiotypically cross-reactive proteins for the detection of idiotypic cross-reactions has been well documented by Kunkel and his co-workers (8). It was not possible to employ this approach here, however, because none of the 50 antisera from related rabbits gave a detectable

cross-reaction with antibody from the proband rabbit 3412. This approach could be used, however, to search for cross-reactions utilizing the proband antibody 2690.

In a previous study which employed idiotypic antisera prepared in guinea pigs (1), it was shown that idiotypic cross-reactions were observed between the Group C antibodies of two siblings, 2459 and 2690. The two antibodies had the same allotypes, gave identical light chain banding patterns in alkaline urea disk gels, and eluted from affinity columns at the same position.

A further comparison of these antibodies was undertaken with anti-2690 idiotypic sera prepared in rabbits. Because the RBI test was not influenced by anti-IgG's it was used to establish the extent of the cross-reaction between these two similar antibodies. Fig. 3 depicts binding curves of [^{125}I] 2690 antibody, 2459 antibody, and a2/b4 pooled IgG to ECF anti-2690. It can be seen that

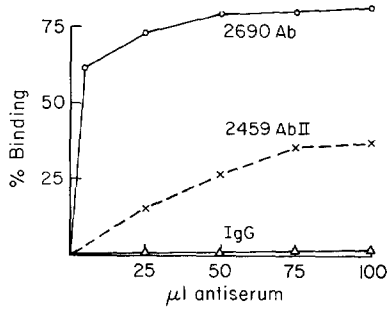

FIG. 3. Binding of [^{125}I] 2690 Ab (○), [^{125}I] 2459 Ab (×--×), and ^{125}I-pooled a2/b4 IgG (△) to increasing amounts of ECF solidified anti-2690.

85% of the 2690 antibody, 35% of the 2459 antibody, and virtually none of the a2/b4 pooled IgG was bound to the antiserum.

Next, unlabeled 2459 and 2690 antibodies and the a2/b4 IgG pool were tested for their ability to inhibit the binding of [^{125}I] 2690 antibody to the anti-2690. The upper frame of Fig. 4 shows that while unlabeled 2690 antibody is an efficient inhibitor of the binding, the reaction was not inhibited by either 2459 antibody or the a2/b4 IgG pool. However, the lower frame of Fig. 4 shows that when the cross-reacting 2459 antibody was substituted for the 2690 antibody, either unlabeled 2690 antibody or 2459 antibody were equally effective as inhibitors of the binding to the ECF anti-2690. No inhibition was seen with the a2/b4 IgG pool. These data indicate that rabbit 2459 produced a Group C antibody component with similar but not identical idiotypy to that of rabbit 2690.

Inhibition of the radiobinding of [^{125}I] 2459 antibody to ECF anti-2690 was next used to detect idiotypic cross-reactions among Group C antibodies from this inbred rabbit family. 15 antisera were tested. Nine were positive and

six were negative for this idiotype. All were negative when 2690 antibody was used as the radiolabeled antibody. All preimmune sera were also negative as inhibitors of this reaction.

Detection of Idiotypic Cross-Reactions by the HI Technique.—Because of limitations in the amount of reagents, the HI technique was used for an extensive search for idiotypic cross-reactions among more than 300 Group C antisera

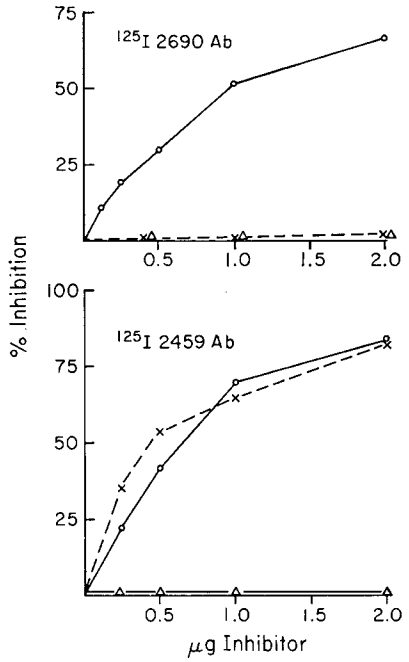

FIG. 4. Upper frame: inhibition of the binding of [^{125}I] 2690 Ab to ECF solidified anti-2690 by unlabeled 2690 (○), 2459 (×), and pooled a2/b4 IgG (△). Lower frame: inhibition of the binding of [^{125}I] 2459 Ab to ECF anti-2690 by unlabeled 2690 (○), 2459 (×), and pooled a2/b4 IgG (△).

as well as the preimmune sera. The HI test used about one one-hundredth the amount of idiotypic antiserum required in the RBI technique.

Before screening experiments were begun, the HI test was used to compare the specificity of the reactions between anti-2690 idiotype and 2690 antibody to that of the reaction between anti-2690 idiotype and 2459 antibody. The results of these experiments are given in Table I. It can be seen that a high concentration of a2/b4 pooled IgG did not inhibit either reaction. While the 2690 antibody inhibits either reaction with equal facility, 2459 antibody has no inhibitory effect on the reaction of 2690 antibody and anti-2690 idiotype. A purified Group C antibody from rabbit 3413 was not inhibitory, although the

light chain had an amino terminal sequence identical for 20 residues to that of antibody 2690. Thus, the data on specificity of the reaction as measured by the HI test are consistent with those obtained using the RBI test.

The heavy and light chains of 2690 antibody were tested as inhibitors of either reaction in order to ascertain whether the idiotypic determinants were composed of sites on both chains or were located on either the light chains or heavy chains. The data of Table I indicate that detection of this idiotype by the HI test requires a specific heavy-light chain combination. The fact that large excesses of isolated chains will inhibit the HI test may be attributable to the contamination of the preparations with intact heavy-light pairs. Such contamination at a level of about 0.5% could cause the inhibition seen here.

TABLE I

Specificity of Anti-2690 Idiotype Measured by HI Test Using RBC's Coated with Either 2690 Ab or 2459 Ab

Inhibitor	Concentration to inhibit hemagglutination*	
	Anti-2690, 2690-RBC	Anti-2690, 2459-RBC
	μg/ml	
a2/b4 pooled IgG	>24,000	>24,000
2690 Ab	1	1
2459 Ab	>2,400	1
3413 Ab‡	>1,000	>1,000
2690 L*	300	375
2690 H*	325	200
2690 H and L	33	12
2690 H+3413 L	333	125
3412 H+2690 L	160	30

* All concentrations are given in μg/ml IgG. The H and L chain concentrations are adjusted accordingly. The greater than sign (>) indicates that no inhibition was seen at the given concentration.

‡ 3413 Ab has allotype a3/b4. Both 2459 Ab and 2690 Ab are a2/b4.

There appears to be no doubt that the idiotype of 2690 is related to the immune response to the Group C carbohydrate. No idiotypic cross-specificity could be obtained in any of the preimmune antisera from the rabbits tested. Furthermore, this idiotype was undetected in the antisera from 25 rabbits of allotype a2/b4, all immunized with Group A streptococci. Finally, the antibodies with this idiotypic determinant were specifically absorbed from Group C antisera with the Group C vaccine, but were not absorbed with the Group A vaccine.

Addition of Group C streptococcal antisera that contained high levels of anti-IgG's to the RBC coated with 2459 antibody gave no interference in the test if the antisera were first diluted 1:10 before addition. With this precaution, the HI test could be used as a screening test for the detection of idiotypic cross-reactions.

Table II lists the presence of the idiotype of antibody 2690 in three groups of rabbits according to their relationship to the proband rabbit 2690. 60% of 80 rabbits directly related to the proband exhibited cross-reactions, while approximately 40% of 53 partially related rabbits expressed this idiotypic cross-specificity. Among the directly related rabbits two had greater than 20% of their IgG expressing the 2690 idiotypic cross-specificity. Approximately 1% of the nonrelated rabbits produced antibody with cross-specificity to the 2690 idiotype. The majority of rabbits in the nonrelated group were from colonies other than the one at The Rockefeller University.

Table III shows a further breakdown of the rabbits which were directly related to the proband 2690. It should be pointed out that all rabbits, in the directly related group, are progeny of the breeding pair listed on the table as

TABLE II

*Detection of Idiotypic Cross-Specificity by HI Employing a Cross-Reacting Antibody as RBC Coat**

Relationship to proband rabbit 2690	Number of rabbits			
	Total	Expressed level of idiotypic cross-specificity‡		
		>2%	0.1–1%	Undetectable
Directly related§	80	14	33	33
Partially related‖	53	2	21	30
Unrelated	97	0	1	96

* The antibody used was from a sibling of the proband 2690.

‡ Method for calculating level of cross-specificity is given in Methods section.

§ This group is further subdivided with respect to relationship to the proband in Table III and by allotype in Table IV.

‖ This group comprises F_1 and F_2 offspring from crosses of 2690 siblings to random bred rabbits.

TABLE III

Relationship to Proband of Directly Related Rabbits Tested for Idiotypic Cross-Specificity*

Relationship to proband rabbit 2690	Number of rabbits			
	Total	Expressed level of idiotypic cross-specificity		
		>2%	0.1–1%	Undetectable
Parents	2	1	—	1
Siblings	17	4	7	6
F_1 offspring‡	34	4	15	15
Other§	27	4	11	12

* All rabbits listed were offspring of the two parents. All rabbits were immunized with Group C streptococci. This represents a further breakdown of the directly related group listed in Table II.

‡ These are offspring from crosses of 2690 with his siblings.

§ This group comprises F_1 anf F_2 offspring from brother-sister crosses of 2690 siblings.

parents. The siblings group includes rabbits 2459 and 2690. The F_1 offspring group are progeny from matings of the buck 2690 to his sisters. All other rabbits produced in other brother-sister and subsequent breedings of this family are listed in a group termed "other related". There is no preferential expression of the 2690 idiotype among these groups. Further studies are required to define the inheritance patterns of the idiotype and these must consider the variability in idiotypic expression.

The earlier report raised the possibility of associations between idiotypy and allotypy and this was examined in greater detail here. Because the inbred family group contained rabbits of allotypes a2/b4 and a2,3/b4, the F_2 generation contained rabbits with the allotype a3/b4. The number of rabbits in each group a allotypic category is listed in Table IV. Tabulated also are the number of rabbits in each category which exhibited idiotypic cross-reactions. Cross-reactions were common for the offspring having the allotypes a2/b4 and a2,3/b4, but were absent in rabbits having the a3/b4 allotype. Although

TABLE IV

Allotypes of Related Rabbits Tested for Idiotypic Cross-Specificity

Allotype	Number of rabbits			
	Total	Expressed level of idiotypic cross-specificity		
		>2%	0.1-1%	Undetectable
a2/b4	39	6	21	12
a2, 3/b4	30	7	12	11
a3/b4	11	0	0	11

further documentation is required, this result suggests that the idiotype of the antibody 2690 which has the allotype a2/b4 is linked to the H chain allotype a2.

One exception to the association of the 2690 idiotypic specificity with allotype a2 was found among the antisera from the partially related group (Table II). The rabbit that produced the cross-reacting antibody had allotype a3/b4,9. Two other rabbits in this group that lacked allotype b4 also produced cross-reacting antibody. These rabbits were of allotypes a2/b9 and a2,3/b9 respectively. No rabbit in any group that lacked both allotypes a2 and b4 produced idiotypically cross-reacting antibody.

DISCUSSION

Any search for the detection of idiotypic cross-reactions among streptococcal group antibodies from immunized rabbits must take into account the possible influence of anti-IgG's on the idiotypic assay. Inhibition of radioprecipitation, for example, was found unsuitable in this study because the IgM anti-IgG in most Group C antisera masked the detection of cross reactions. Such

interference was not seen with the inhibition of radiobinding assay, and cross-reactions could be detected with this system. The anti-IgG's furthermore, had no effect on the hemagglutination inhibition test. This latter test was most widely employed in the search for idiotypic cross-reactions because this method conserved reagents.

At least two major factors influence the occurrence of idiotypic cross-reactions among groups of antibodies or myeloma proteins with antibody activity. Specificity for the same antigen appears to be a major factor in the occurrence of cross-reactions, although exceptions to this have been reported by Oudin and Cazenave (21). In the studies reported here, no idiotypic cross-reactions were seen between antibodies to Group C carbohydrate and antibodies to Group A carbohydrate. Williams et al. (7) observed idiotypic cross-reactions among those IgM proteins with activity for I antigens on red blood cells and more recently, Kunkel et al. (8) showed cross-specificities among a group of IgM proteins having in common activity against γ-globulins. Carson and Weigert (3) showed that mouse antibodies to α-1,3 dextran and mouse myeloma proteins with activity to this polysaccharide had similar idiotypic specificities. These associations between antibody specificity and idiotypic cross-reactivity strongly indicate participation of the antigen binding sites in the idiotypic marker.

Secondly, the occurrence of idiotypic cross-reactions among antibodies of the same specificity is influenced by genetic factors. Earlier studies with inbred rabbits suggested the inheritance of idiotypic markers of streptococcal Group C antibody (1). Similar studies by Winfield et al. (22) with rabbit pneumococcal antibodies failed to confirm these findings. One possible explanation for the discrepancy between the two studies is that different methods were used to prepare the idiotypic antisera. In the earlier studies with streptococcal antibodies, the idiotypic antisera were prepared in guinea pigs and were subsequently absorbed with pooled rabbit IgG. In the studies with pneumococcal antibodies the idiotypic antisera were prepared in allotypically matched rabbits. The possibility has been raised that the idiotypic antisera prepared in allotypically matched rabbits identify a narrow antigenic marker on the immunoglobulin molecule, whereas the idiotypic antisera prepared in a heterologous species recognize a broad specificity in the variable region (H. G. Kunkel, personal communication). As a consequence, it would not be surprising if an idiotypic antiserum prepared in a heterologous species detected a larger number of cross-reactions than idiotypic antisera prepared in a homologous species.

In the studies reported here, the idiotypic antisera to streptococcal antibodies were prepared in allotypically matched rabbits. Using these antisera idiotypic cross-reactions were commonly observed among Group C antibodies from an inbred rabbit family and rarely observed among the antibodies from unrelated rabbits. For this reason, it appears that differences in the method of preparation of idiotypic antisera is not the explanation for the discrepancy between the findings of the streptococcal system and the pneumococcal sys-

tem. There are at least two other explanations which are more likely. The first is that not all idiotypes are equally expressed in a given family of rabbits or in an inbred strain of mice. The second factor is that the two studies used entirely different methods for the detection of idiotypic cross-reactions.

Variation in the expression of an idiotype among immunized inbred animals has been well documented. In the studies reported here, one idiotype was observed in 58% of the related rabbits, whereas another idiotype was not detected in any of these rabbits. Such differences in expression of idiotypy have also been reported by Eichmann (23) for Group A streptococcal antibodies in inbred mice. One of the idiotypes studied by Eichmann was expressed in 80% of the A/J mice and another idiotype was expressed in less than 20%.

There is one technical aspect concerning idiotypic assays which can have a tremendous influence on the detection of idiotypic cross-reactions. In the studies reported here, no cross-reactions were observed when the RBI test or HI test employed the streptococcal antibody against which the idiotypic antiserum was raised. Cross-reactions were only detected when a second strongly cross-reactive streptococcal antibody was employed. This phenomenon was earlier pointed out by Kunkel et al. (8) in their studies of idiotypic cross-specificities among myeloma proteins with activity for similar antigenic determinants. Using this approach, approximately 60% of rabbits related to the proband produced antibodies with idiotypic cross-specificity, whereas approximately 1% of nonrelated rabbits showed this cross-specificity.

While this is a strong indication for inheritance of determinants of this idiotypic cross-specificity, the exact mode of inheritance is not evident for several reasons. As has already been mentioned, variable expression of the idiotype may obscure patterns of inheritance. Genetic studies are further complicated by the finding that most idiotypic determinants require specific heavy-light chain combinations (24), and heavy and light chain allotypic genes are not linked. In spite of these complications, inheritance of idiotypy has been demonstrated in certain inbred mouse strains (2–5). Furthermore, these idiotypes have been shown to be linked in several instances to the C_H allotype genes (2–4).

Within the family of the proband rabbit studied here, only those rabbits which had allotype a2 produced idiotypically cross-reactive antibodies, whereas the antibodies of the a3/b4 rabbits did not. This suggests linkage between the V_H allotype and this idiotype, but the subject needs further study. In a partially related group of rabbits, for example, one animal produced cross-reactive antibody although its allotype was a3/b4,9. Thus, it cannot be concluded that the gene or genes controlling the synthesis of the idiotype are identical to the genes coding for the V_H allotype. An instance of an identical idiotype on both an a3 and a group a-negative antibody has been previously observed (15).

Because the family of the proband was homogeneous for light chain allotypes, no data concerning the linkage of the L chain to the idiotype were available. However, among the partially related family members, two animals having

the a2 allotype from the 2690 family but lacking b4, showed the presence of antibodies with idiotypic cross-specificity. No rabbit which lacked both a2 and b4 produced cross-reactive antibody.

Perhaps the most perplexing observation in these studies and the studies of others, is the failure to detect cross-reactions by test systems which employ an idiotypic antiserum and the antibody against which it was prepared. This result implies that the *V* regions of these antibodies are quite similar, but are not identical. If a single germ-line gene encodes a complete *V* region, one would expect identical molecules to be inherited. This is not observed, although a number of idiotypically similar antibodies are found in the antisera from the family members. Similar, but not identical, idiotypy would suggest somatic generation of the antibody binding sites as opposed to a strict germ-line inheritance. However, a purely somatic mechanism for generation of binding sites does not explain the familial clustering of cross-specificity. The use of allotypically matched rabbits to prepare anti-idiotypic sera reduced the possibility that subgroup or allotypic differences are involved in these cross-reactions.

Structural comparisons of idiotypically cross-reactive molecules will be necessary to answer these questions. A possible explanation for the idiotypic cross-reactivity observed here is that the antibodies are identical in some, but not all, hypervariable regions. There is some evidence that idiotypic antisera recognize hypervariable regions (8, 25). Genes coding for hypervariable regions could be inherited as episomes as proposed by Wu and Kabat (26). The hypervariable region sequences could then be inserted into discrete predetermined areas of the *V* regions. This type of mechanism would account for the data reported here as well as results obtained in a previous study (15).

SUMMARY

Idiotypic cross-reactions among antibodies to Group C streptococcal carbohydrate were studied using idiotypic antisera prepared in allotypically matched rabbits. Antibodies with idiotypic cross-specificity to one proband antibody were detected in 58% of the antisera from related rabbits, while approximately 1% of nonrelated rabbits produced antibody with this specificity. The cross-specificity was related to the group a (V_H) allotype of 133 rabbits tested with only one exception.

Studies utilizing antisera against a second proband antibody failed to detect antibodies with idiotypic cross-reactivity among the same group of related rabbits. This result emphasizes the variation in expression of idiotypic determinants of antibodies.

It was further shown that the presence of anti-IgG's in the streptococcal antisera interfere with the detection of idiotypic cross-reactions. These anti-IgG's masked the presence of antibodies with idiotypic cross-specificity when inhibition of precipitation tests were used for their detection.

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