THE INHERITANCE OF INDIVIDUAL ANTIGENIC SPECIFICITIES OF RABBIT ANTIBODIES TO STREPTOCOCCAL CARBOHYDRATES*

By KLAUS EICHMANN,‡ M.D., and THOMAS J. KINDT,§ Ph.D.

(From The Rockefeller University, New York 10021)

(Received for publication 23 April 1971)

Selective brother-sister mating of rabbits has revealed that the restriction in the heterogeneity of the antibody response to streptococcal carbohydrates is under genetic control (1). One possible explanation for an inherited limitation of the number of different antibody molecules is that these specially bred rabbits have inherited only a portion of the gene pool which normally codes for a heterogeneous antibody response. If this is the case, structurally identical antibodies should be observed in those rabbit families in which anti-streptococcal antibodies of restricted heterogeneity are a common event, because in these families only a limited number of gene products are available for antigenic selection. Discovery of antibodies with identical individual antigenic specificities in several generations of these rabbit families would support this hypothesis.

The term individual antigenic specificity (IAS)[1] describes the antigenic individuality of a monoclonal immunoglobulin (2), that is recognized by anti-antibody raised in a heterologous species. Kunkel and associates (2, 3) and subsequently other investigators (4, 5) have demonstrated that the determinants responsible for this antigenic individuality are localized in the Fab portion. The variable region sequences of either the H or the L chain, or a combination of both, determine the antigenic uniqueness of each antibody (3, 5). A similar antigenic uniqueness of specific antibodies, termed idiotypy, is recognized by anti-antibody prepared in allotypically matched animals of the same species (6–8). The term cross-specificity was introduced by Williams et al. (9) for an IAS which is shared by immunoglobulins with specificity for the same antigen and isolated from different individuals.

The evidence presented here suggests that IAS is a genetically transmitted character of an antibody. An unexpectedly high proportion of antibodies with

* This work was supported by the National Institutes of Health grant AI 08429 and by a grant-in-aid from the American Heart Association. The work was sponsored by the Commission on Streptococcal and Staphylococcal Diseases of the Armed Forces Epidemiological Board, and was supported in part by the U. S. Army Medical Research and Development Command, under research contract number DADA 17-67-C-7043.

‡ Present address: Institut fur Genetik, University of Koln, 5 Koln, West Germany.

§ Established investigator, American Heart Association.

[1] *Abbreviations used in this paper:* IAS, individual antigenic specificity; RBC, red blood cells; V, variable.

cross-specificity has been found in several generations of a rabbit family which has a high incidence of monoclonal-like responses to streptococcal Group C carbohydrate (1). No cross-specificity was found among the antibodies of non-related rabbits. Furthermore, a given IAS appears to be associated with a given allotypic combination.

Materials and Methods

Preparation of Streptococcal Vaccines and Streptococcal Antigens, Immunization Procedures, Electrophoretic Procedures, Immunological and Immunochemical Procedures.—These procedures have been described previously (4, 10–13).

Rabbits.—Rabbit families obtained through selective brother-sister mating were used in these studies. The principles of selection and mating have been described previously (1).

Purification of Specific Antibody from Streptococcal Antisera.—Antibodies to the group-specific streptococcal carbohydrates were isolated by affinity-immunoabsorbent chromatography, followed by preparative agarose electrophoresis. Isolation and simultaneous fractionation of antibodies by these procedures have been described in detail previously (14).

Purification of Preimmune IgG.—Preimmune IgG was isolated by two subsequent precipitations of serum with 1.4 volumes of 3 M ammonium sulfate, followed by diethylaminoethyl (DEAE)-cellulose chromatography, as described by Levy and Sober (15).

Radioactive Labeling of IgG.—Purified rabbit antibodies and IgG were labeled with ^{125}I according to the method of McFarlane (16).

Preparation of Individually Specific Antisera.—Antisera to isolated antibody components were prepared in Rockefeller strain guinea pigs. The guinea pigs were rendered immune tolerant to common IgG determinants by intravenous injection of 5 mg pooled rabbit IgG (Pentex Biochemicals, Kankakee, Ill., Fx II) as described by Henney and Ishisaka (17). On the same day, a total of 100 μg purified and antigen-free antibody was mixed with complete Freund's adjuvant and was given in the footpads. 4 wk later the guinea pigs were bled out. All antisera were subsequently passed through immunoabsorbent columns, prepared from Sepharose and pooled IgG by the cyanogen bromide procedure (18). The dimensions of the columns were chosen so that each milliliter of antiserum was absorbed by an amount of immunoabsorbent which contained 20 mg IgG.

Quantitative Radioprecipitin and Inhibition Test with Guinea Pig Anti-Rabbit Antisera.—The equivalence point for each guinea pig antiserum with its homologous antibody was determined by a reverse quantitative precipitin test. The guinea pig antisera were diluted with an equal volume of normal guinea pig serum before use. To a series of tubes containing 5 μg of the homologous radiolabeled antibody, increasing amounts of 25–150 μl antiserum were added. The total volume in each tube was diluted to 200 μl with PO_4 buffer. The amount of precipitate was measured after incubation for 2 hr at room temperature and for 48 hr at 4°C by counting the radioactivity in precipitates and supernatants.

For the quantitative inhibition test, a point of slight antigen excess was chosen from the quantitative precipitin curve. To a series of tubes containing equal amounts of antiserum, increasing amounts of cold preimmune IgG or antibodies were added as inhibitors. After 30 min, 5 μg of the radiolabeled homologous antibody was added to each tube. Further processing was done as described for the quantitative precipitin test.

Hemagglutination-Inhibition Test.—Inhibition of passive hemagglutination was performed as described by Kunkel (19), using human blood group O red blood cells (RBC) which had been coated with rabbit antibody preparations by the chromium chloride procedure (20). Hemagglutination was carried out with guinea pig antisera and their homologous antibodies coated to RBC. The highest dilution of each guinea pig antiserum which still gave visible

hemagglutination was determined. At this dilution preimmune rabbit IgG in various concentrations was added to the test to inhibit hemagglutination. The plates were read after incubation for 1 hr at room temperature.

Determination of Antibody Activity in Preimmune IgG.—Antibody activity in preimmune IgG was determined by the modified Farr technique (21), using the soluble carbohydrate of Group C streptococci. The carbohydrate was labeled with ^{125}I after cyanogen bromide incorporation of tyramine (E. C. Gotschlich, unpublished method). To determine the binding capacity of Group C antibodies, equal amounts of 100 ng carbohydrate were mixed with 10–50 μl of Group C antisera which were diluted in heated fetal calf serum to a concentration of 30 μg antibody/ml. To determine the binding capacity of preimmune IgG, 50 μl of undiluted preimmune rabbit sera were used which contained 3–5 mg IgG/ml. The amount of antigen bound was determined after precipitation with 50% ammonium sulfate by counting the radioactivity in precipitates and supernatants.

Radio Disc Electrophoresis of Reduced and Alkylated Antibody—Anti-Antibody Precipitates.—20 μg of radiolabeled antibody was precipitated with an equivalent amount of guinea pig antiserum. The precipitate was washed and redissolved in 7 M guanidine-HCl. Reduction, alkylation, and alkaline polyacrylamide disc electrophoresis in urea followed the procedure of Reisfeld and Small (22). Electrophoresis was carried out for 5 hr at 2.5 ma/tube so that the small pore gel (6.5 cm) contained both H and L chains. After fixation in 10% trichloroacetic acid (TCA), the gels were sliced into 1.5 mm slices, and the radioactivity in each slice counted.

Preparation of Anti-Allotype Antisera.—These antisera were prepared in rabbits according to the principles of Oudin (23). Several anti-allotype sera were obtained from Dr. C. W. Todd.

Determination of Allotypes.—Determination of the allotypes of each rabbit employed the interfacial precipitin test. Quantitative radioprecipitation of isolated antibody components by anti-allotype antisera was performed as previously described (24). Determination of d11 and d12 markers on some preparations was performed by the hemagglutination-inhibition test of Dr. C. W. Todd. On others, these markers were determined by cyanogen bromide digestion and subsequent gel filtration on G-200 Sephadex, as previously described (25).

RESULTS

An exceptionally high proportion of monoclonal-like antibody responses to Group C streptococcal carbohydrate has been observed in certain selectively bred rabbit families (1). One of these families consisted of 2 parental rabbits, 25 F_1 generation rabbits, and 15 F_2 generation rabbits. All had been immunized with Group C vaccine. Selection for inbreeding was on the basis of restriction in antibody heterogeneity. To trace the inheritance of antibodies with shared individual antigenic specificity, four rabbits of this family with uniform antibody responses were chosen to serve as probands. Their antibodies are referred to as proband antibodies. Guinea pig antisera detecting the IAS of these proband antibodies were then tested for cross-reactivity with Group C antibodies and preimmune IgG from related and nonrelated rabbits. Two types of cross-specificities have been observed in these experiments, which are referred to as precipitating and nonprecipitating cross-specificity. The term precipitating cross-specificity is used for the shared IAS of the antibodies from different individuals, as detected by direct precipitin analysis. The term nonprecipitating

cross-specificity designates the cross-reactivity of individually specific antisera with IgG from other rabbits, as detected by precipitin-inhibition analysis. For the sake of convenience, the experimental data which are concerned with precipitating and nonpreciitating cross-specificity are described in separate sections.

Precipitating Cross-Specificity.—In an initial screening procedure, antisera against the proband antibodies were tested for cross-precipitation with the Group C antibodies from the 42 members of the family, and with the Group C antibodies from 48 unrelated rabbits. This was done by agar immunodiffusion

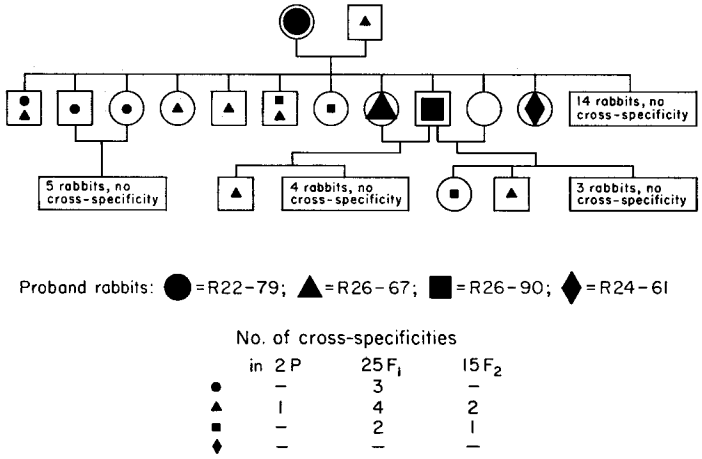

FIG. 1. Pedigree of a rabbit family consisting of 2 parental, 25 F_1, and 15 F_2 generation rabbits. Large symbols: rabbits from which the proband antibodies were isolated. Small symbols: rabbits which produced antibodies with precipitating cross-specificity for a proband antibody. Squares, males; circles, females. All rabbits were immunized with Group C vaccine.

techniques, which employed the hyperimmune sera of each rabbit. No cross-precipitation was observed with the antibodies from the 48 unrelated rabbits, whereas the antisera against 3 of the 4 proband antibodies cross-precipitated with antibodies from several members of the family. This is demonstrated in the pedigree of this family in Fig. 1. Each of the four individual specificities is represented by one of the black symbols. The rabbits from which the proband antibodies were isolated are indicated by the large symbols, and the rabbits which produced antibodies with precipitating cross-specificity are indicated by the small symbols. For example, the guinea pig antiserum which identified the IAS of the proband antibody indicated by the large triangle gave cross-precipitation with the antibodies from 1 of the 2 parents, 4 of the 25 F_1, and 2 of the 15 F_2 generation rabbits. Cross-precipitations of the antisera against the two

proband antibodies which are indicated by the circle and the square were less frequently observed. No cross-precipitation was observed with the antiserum against the proband antibody indicated by the diamond.

Antibodies were then isolated from those antisera shown by this survey to contain antibodies which shared the IAS of a proband antibody. Immunologic relationships among these antibodies were examined by immunodiffusion

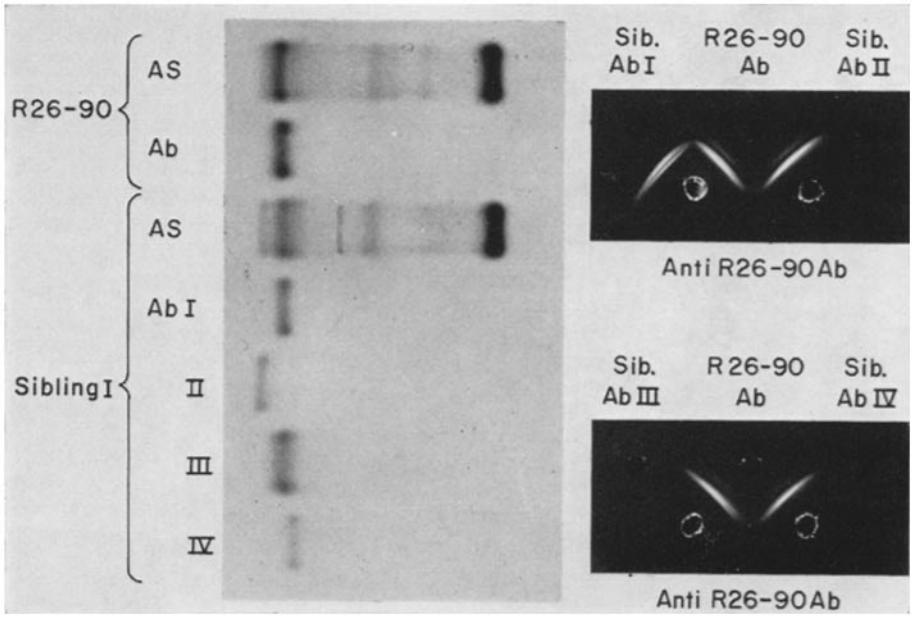

FIG. 2. Left frame: microzone electrophoretic patterns of the antisera (AS) and isolated antibody components (Ab) from proband rabbit R26-90 and from Sibling 1. Right frame: immunodiffusion analysis of the antibody components depicted in the left frame with a guinea pig antiserum to R26-90Ab (anti-R26–90Ab). All antibodies have been applied in equal concentrations of 1 mg/ml.

analysis. The experiments depicted in Fig. 2 demonstrate the cross-specificity of the antibodies isolated from a pair of siblings which belong to the F_1 generation. Depicted in the left frame are the microzone electrophoretic patterns of the antisera and isolated antibodies from both rabbits. Proband rabbit R26-90 is indicated by the large square in Fig. 1, and antisera prepared in three guinea pigs cross-precipitated with the antibody of sibling 1. Affinity chromatography fraction (14) of the sibling's antibody yielded four distinct components, which are referred to as Ab I–IV. The relationships between R26-90Ab and Ab I–IV are revealed by the immunodiffusion experiments shown in the right frame. Only Ab I is precipitated by anti-R26–90Ab, whereas Ab II–IV show no cross-

precipitation. A line of identity is formed between Ab I and R26-90Ab, suggesting identical IAS of both antibodies. It should be emphasized that both R26-90Ab and sibling 1 Ab I have the same electrophoretic mobility and the same relative binding affinity (14) for an immunoabsorbent column.

The experiments depicted in Fig. 3 trace an IAS through three generations of this family. Depicted in the left frame of Fig. 3 are the microzone electrophoretic patterns of the antisera and isolated antibodies from three rabbits

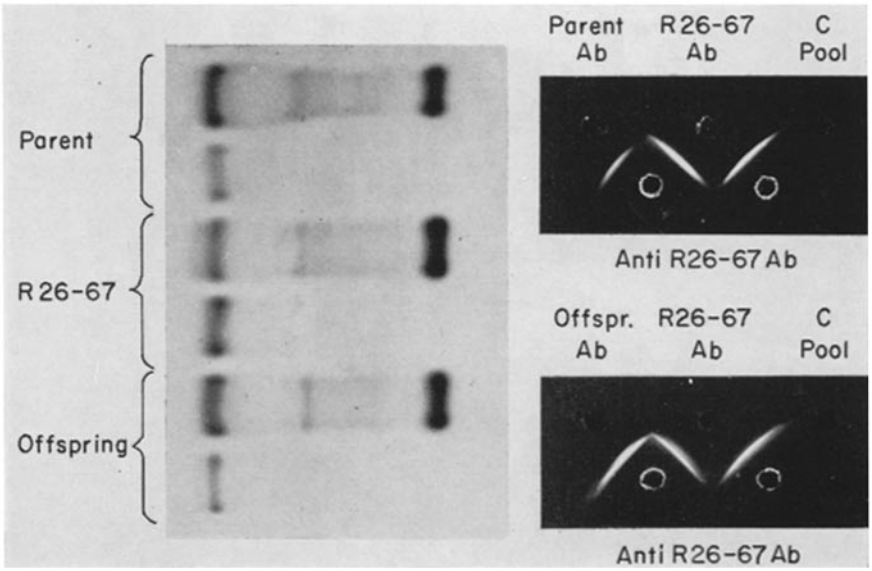

FIG. 3. Left frame: microzone electrophoretic patterns of the antisera and isolated antibody components from proband rabbit R26–67, from its father, and from its offspring. Right frame: immunodiffusion analysis of the antibody components depicted in the left frame with a guinea pig antiserum to R26–67Ab (anti-R26–67Ab). C pool: pool of Group C antibodies from five unrelated rabbits. All antibodies have been applied in equal concentrations: 1mg/ml.

selected from the parental, F_1, and F_2 generations of the family. Proband rabbit R26-67 is indicated by the large triangle in Fig. 1, and anti-R26–67Ab cross-precipitated with the antibodies from its father and its offspring. Ouchterlony experiments to compare the parents' and the offsprings' antibody to R26-67Ab are depicted in the right frame of Fig. 3. Included as a control in each plate is a pool of Group C antibodies from five unrelated rabbits. A line of identity is formed between the offspring's antibody and R26–67Ab, suggesting identical IAS. In contrast, the precipitin line of the parent's antibody does not fuse with that of R26–67Ab, suggesting partial identity. Nevertheless, these studies trace at least a component of this IAS through three generations

of this family. Again, all these antibodies with cross-specificity have the same electrophoretic mobility and the same relative binding affinity for an immunoabsorbent column.

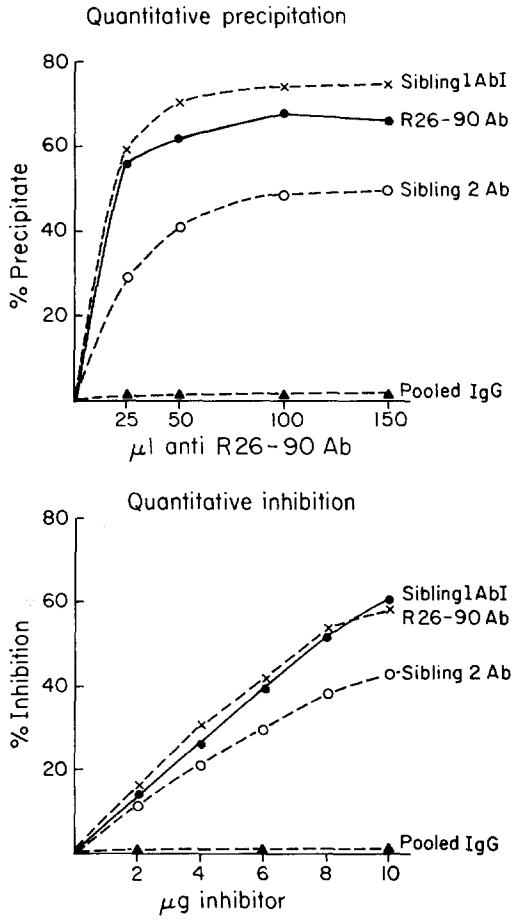

FIG. 4. Top frame: quantitative radioprecipitin test performed with 5 μg radiolabeled R26–90Ab, sibling 1 Ab I, sibling 2 Ab, and pooled rabbit IgG, using increasing amounts of anti-R26–90Ab. Bottom frame: quantitative radioprecipitin inhibition test performed with 5 μg radiolabeled R26–90Ab and 25 μl anti-R26–90Ab. Increasing amounts of the cold antibodies were added as inhibitors.

Cross-specificity among these antibody preparations has been examined quantitatively by radioprecipitin and inhibition experiments, and a comparison by quantitative precipitin is shown in Fig. 4. Depicted in the top frame of Fig. 4 are the quantitative radioprecipitin data obtained with anti-R26–90Ab and R26-90Ab. Also shown are the quantitative precipitin curves obtained

with the same antiserum and Ab I from sibling 1, which showed identical cross-specificity by immunodiffusion (Fig. 2), and the antibody of sibling 2, which showed only partial identity (not shown in Fig. 2). Sibling 1 Ab I is precipitated to the same, if not greater, extent than is R26–90Ab. In contrast, sibling 2 Ab is precipitated to a lesser extent. Electrophoretic data suggest sibling 2 Ab is predominantly a single molecular species, so the partial cross-reaction with anti-R26–90Ab is a reflection of similar but not identical IAS determinants. Pooled rabbit IgG, included as a control, is not precipitated by anti-R26–90Ab.

Depicted in the lower frame of Fig. 4 are quantitative inhibition experiments which employed R26–90Ab, sibling 1 Ab I, and sibling 2 Ab as inhibitors of the precipitin reaction between anti-R26–90Ab and R26-90Ab. The inhibition curves obtained with R26–90Ab and with sibling 1 Ab I are identical. Sibling 2 Ab inhibits to a somewhat lesser degree. Greater inhibition of the precipitin reaction was not achieved by increasing the amount of sibling 2 Ab added to the reaction. This is another indication that sibling 2 Ab does not possess all of the IAS determinants of R26-90Ab. From these inhibition experiments it is possible to determine the amount of an inhibitor which is required to achieve 50% inhibition. The I_{50} value for each inhibitor is then calculated by dividing this amount by the quantity of proband antibody required to obtain 50% inhibition. Thus the I_{50} value of the proband antibody is 1 by definition, and an I_{50} value of 1 for an inhibitor is an indication that its IAS has complete serological identity to the proband antibody. The I_{50} value for sibling 1 Ab I is 0.98 and that for sibling 2 Ab is 1.55. These data suggest that R26–90Ab and Ab I of sibling 1 have identical IAS, whereas sibling 2 Ab possesses only a portion of this IAS.

It is evident from these observations on either complete or partial cross-specificity that antibodies may share either all their individually specific determinants or only a portion of them. Comparison of the electrophoretic mobilities of the polypeptide chains of antibodies with cross-specificity provides another parameter for distinguishing between complete and partial identity. The H and L chain mobilities of such antibodies were compared by a radio disc electrophoresis assay, which employed the radiolabeled antibodies after precipitation with the guinea pig antisera. By this means, only those L and H chain bands appear on the gels which were derived from antibody molecules identified by the antiserum. Minor antibody components lacking the IAS, which are present in each antibody preparation, are eliminated. The left frames of Fig. 5 contain the disc electrophoresis patterns obtained with R26–67Ab (top), the offspring's Ab (middle), and the mixture of both (bottom). One major H chain band and one major L chain band is observed in both antibodies. Disc electrophoresis of a mixture of both antibodies shows that the mobilities of H and L chains of each antibody are identical. As shown in Fig. 3, these two antibodies had complete cross-specificity. The right frames of Fig. 5 contain the patterns

obtained with R26–67Ab (top), the parent's Ab (middle), and the mixture of both (bottom). In this case only the L chain bands of both antibodies had identical electrophoretic mobilities. A difference in electrophoretic mobility of the H chain bands is noted. This is shown by the fact that the chains in the mixture of both antibodies migrate within several segments of the gel and not

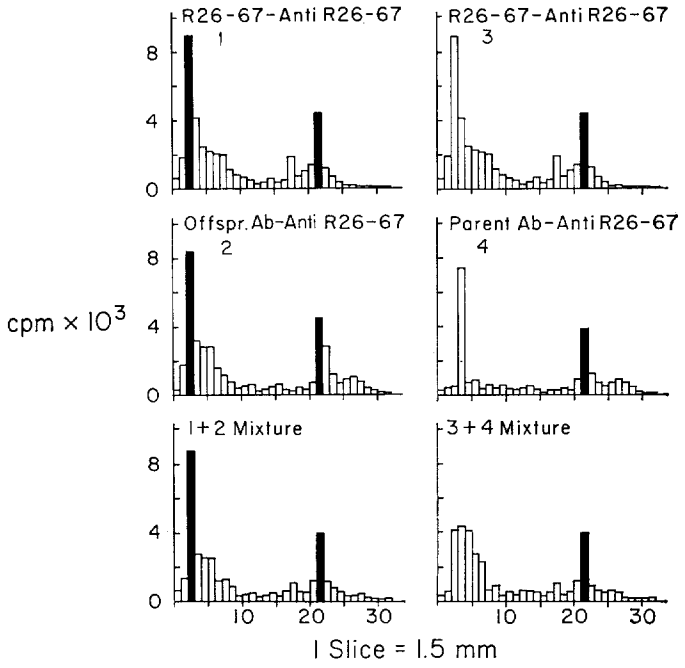

FIG. 5. Radio disc electrophoresis of reduced and alkylated precipitates obtained with anti-R26–67Ab and 20 μg of the radiolabeled antibodies from R26–67 (gels 1 and 3), from its offspring (gel 2), and from its father (gel 4). The experiments depicted at the bottom were done with mixtures of the samples run on gels 1 and 2, and on gels 3 and 4, respectively. Slices 1–15 contain the H chains and slices 16–32, the L chains. H and L chain bands of identical electrophoretic mobility are indicated by the black bars.

within one segment. As shown in Fig. 3, these two antibodies had only partially identical cross-specificities. These electrophoretic data suggest that antibodies with partially identical cross-specificity may share determinants on one polypeptide chain, but have different determinants on the other polypeptide chain.

The rabbit family under study here had the allotypes a2, a3, and b4, and the members of the family were either homozygous a2, a2, or heterozygous a2, a3 for these H chain markers. The linkage between group a and group d markers was a2-d12, and a3-d11 (26). Uniform antibodies with an a3 H chain were a rare event in this family, and all four proband antibodies consisted predomi-

nantly of a2-b4 molecules. All purified antibody components which shared the IAS with these proband antibodies also consisted primarily of a2-b4 molecules, and none of the rare a3-b4 antibodies in the family shared precipitating cross-specificity with these proband antibodies. This suggests, but does not prove, a linkage between allotypic markers and IAS.

To exclude the possibility that the cross-specificity detected by the guinea pig antisera was influenced by allotypic determinants, comparative quantitative precipitation by anti-allotype and anti-IAS antisera was performed with the proband antibodies and allotypically matched antibody preparations. Antibodies were selected which did and did not have precipitating cross-specificity, but had identical allotypes. As shown in Table I, R26–90Ab and Ab I from

TABLE I

Comparative Quantitative Precipitation by Anti-Allotypic and Anti-IAS Antisera

Antibody or IgG preparations (allotype)*	Per cent precipitated by anti-allotype antibody			Per cent precipitated by anti-R26–90Ab‡
	anti-a2	-a3	-b4	
	(%)	(%)	(%)	(%)
R26-90 (a2, a3, b4)				
Ab, Group C	66	14	98	68
Sibling 1 (a2, a3, b4)				
Ab I, Group C	71	1	97	74
Ab III, Group C	71	0	99	1
IgG (a2, b4)	60	0	96	4

* Determined by interfacial precipitin test with preimmune serum.

‡ Determined by quantitative radioprecipitation of the isolated antibody components.

sibling 1 consisted primarily of a2-b4 molecules, and both were precipitated by anti-R26–90Ab. However, Ab III from sibling 1 (see Fig. 2), which was also a2-b4, and pooled a2-b4 IgG were not precipitated by anti-R26–90Ab. Although R26–90Ab contained a minor component consisting of a3 molecules, the data indicate that the a2 antibody population in R26–90Ab is the major component which is precipitated by anti-R26–90Ab. The lack of precipitation of this antiserum with heterologous a2 preparations clearly shows that the antisera used in these experiments detect individual specificities and not allotypic specificities. Furthermore, the data obtained with both allotypic and individually specific precipitation indicate that sibling 1 Ab I is a more highly purified preparation of an antibody with this specificity than is R26–90Ab.

The experiments described thus far can be summarized as follows: (*a*) The IAS of the Group C antibodies from 3 proband rabbits were also observed in the Group C antibodies in as many as 7 of 42 related rabbits, but in none of the Group C antibodies from 48 unrelated rabbits. (*b*) The cross-specificity of

antibodies from related rabbits may be either partial or complete, as judged by immunodiffusion and radioprecipitin analysis. In the case of complete identity, the electrophoretic mobilities of the antibodies and those of their H and L chains are indistinguishable. In the case of partial identity, the electrophoretic mobilities of one of the polypeptide chains are identical, but those of the other chain are different. (*c*) The antisera used to detect shared IAS did not recognize allotypic determinants. Antibodies with shared IAS, however, had identical allotypes.

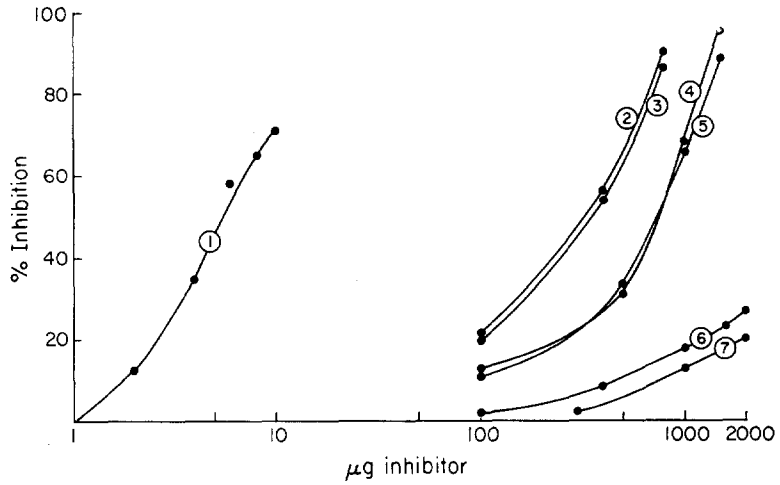

FIG. 6. Quantitative radioprecipitin-inhibition test performed with 5 μg radiolabeled R26-90Ab and 25 μl anti-R26–90Ab. Increasing amounts of cold inhibitors were added, and they were as follows: curve 1, R26–90Ab; curve 2, R26–90 IgG; curve 3, sibling 1 IgG; curve 4, sibling 3 IgG; curve 5, R11 IgG; curve 6, R8 IgG; curve 7, R7 IgG. I_{50} values of these IgG preparations are listed in Table II.

Nonprecipitating Cross-Specificity.—Nonprecipitating cross-specificity has been demonstrated by Kunkel and associates by their observation that the precipitation of a myeloma protein with its individually specific antiserum is inhibited by large excesses of heterologous IgG (2, 3). By the use of the radioprecipitin inhibition test, guinea pig antisera against the proband antibodies were tested for reactivity with antibody preparations and preimmune IgG of related and nonrelated rabbits. Each preimmune IgG preparation inhibited to some extent, but marked variations in the degree of inhibition were noted.

Depicted in Fig. 6 are the quantitative inhibition curves obtained when the precipitin reaction of R26–90Ab and anti-R26–90Ab was inhibited by preimmune IgG preparations from various sources. Because the concentrations of the different IgG preparations required to obtain 50% inhibition covered such

a wide range, these inhibition curves are plotted on semilogarithmic coordinates. Included for comparison is the inhibition achieved when the proband antibody was employed as inhibitor (curve 1). Curve 2 was obtained with preimmune IgG of rabbit R26–90. Curve 3 was obtained with preimmune IgG from sibling 1, which developed an antibody with identical precipitating cross-

TABLE II

I_{50} Values of Various Preimmune IgG and Antibody Preparations as Inhibitors of the R26–90Ab-anti-R26–90Ab Precipitin Reaction*

	Source and characteristics of inhibitor (Allotype)	I_{50} (R26–90Ab:1)	Excess over R26-90 IgG
Preimmune IgG from			
Related rabbits	R26–90 (2, 3, 4, 11, 12)	64	1
	Sib 1 (2, 3, 4, 11, 12)	64	1
	Sib 2 (2, 4, 12)	70	1
	Sib 3 (2, 3, 4, 11, 12)	116	2
	Sib 4 (2, 4, 12)	122	2
Nonrelated rabbits	R11 (2, 4, 12)	113	2
	Pool (1, 2, 3, 4, 5, 11, 12)	181	3
	R12 (3, 4, 12)	250	4
	R13 (2, 9, 12)	280	4
	R8 (1, 3, 4, 11, 12)	640‡	10
	R14 (1, 5, 12)	800‡	12
	R15 (1, 4, 12)	1240‡	20
	R7 (1, 4, 12)	1800‡	30
Group C antibody from			
Related rabbits	Sib 3: restricted Ab (2, 3, 4, 11, 12)	78	
	Sib 4: restricted Ab (2, 4, 12)	92	
Nonrelated rabbits	R8: heterogeneous Ab (1, 3, 4, 11, 12)	245	
	R7: uniform Ab (1, 4, 12)	3000‡	

* The allotype of R26–90Ab is a2, b4, d12.

‡ These I_{50} values were obtained by extrapolation of the inhibition curves.

specificity (see Figs. 2 and 4). Curve 4 was obtained with the preimmune IgG of sibling 3, which did not develop an antibody with precipitating cross-specificity. Curves 5, 6, and 7 were obtained with preimmune IgG from nonrelated rabbits of various allotypes.

I_{50} values as defined earlier for these and other IgG preparations are listed in the upper part of Table II. The various preparations have been used as inhibitors of the precipitin reaction between anti-R26–90Ab and R26–90Ab. Preimmune IgG of rabbit R26–90 and of siblings 1 and 2 were the strongest inhibitors, and these rabbits developed antibodies with precipitating cross-

specificity (Figs. 2 and 4). On the other hand, preimmune IgG preparations of siblings 3 and 4 were about one-half as effective as inhibitors and the antibodies developed by these siblings did not share the IAS of the proband antibody. Use of preimmune IgG from nonrelated rabbits as inhibitors revealed a wide range of inhibitory capacities. To insure that IgG preparations from rabbits clearly unrelated to the proband rabbit were included in the test, rabbits (R11–R15) were obtained from a colony at the City of Hope National Medical Center in California. Preimmune IgG from one of these rabbits, R11, was as effective an inhibitor as was preimmune IgG from siblings 3 and 4. It is significant that R11 shared the allotypes a2, b4, and d12 with the proband antibody. Pooled IgG with all group a allotypes was three times less effective than was the proband's IgG. IgG preparations from rabbits R12 and R13 were four times less effective than was the proband's IgG, and both shared the allotype of one chain but not that of the other chain with the proband antibody. IgG preparations from four rabbits lacking the a2 marker (R8, R14, R15, and R7) were 12–30 times less effective as inhibitors, regardless of whether the b marker was or was not shared with the proband antibody. In these cases 50% inhibition was not achieved with a 400-fold excess, so the I_{50} values, marked by double daggers, were calculated by extrapolation of the inhibition curves. It was necessary to do so because IgG concentrations higher than 400-fold excess were not possible in this test system.

It is evident from these data that preimmune IgG from the proband rabbit (R26–90) and from several of its relatives (siblings 1 and 2) was two times as effective an inhibitor as was the preimmune IgG from other relatives (siblings 3 and 4) and from unrelated rabbits with the same group a allotype (R11). It is noteworthy that siblings 1 and 2 developed antibodies with precipitating cross-specificity for R26–90Ab (see Figs. 2 and 4), whereas siblings 3 and 4 did not. Furthermore, it appears that the IAS of R26–90Ab is preferentially associated with the a2:b4 allotypic combination.

In the lower part of Table II are the I_{50} values obtained when Group C antibodies which did not exhibit precipitating cross-specificity were employed as inhibitors. The antibodies of siblings 3 and 4 were of moderately restricted heterogeneity, and their inhibitory capacity was only slightly higher than that obtained with their preimmune IgG (top of Table II). In contrast, the antibody of R8 was 2.5 times more inhibitory than was its preimmune IgG and the antibody of R7 was very much less inhibitory than was its IgG. This is consistent with the electrophoretic behavior of these two antibody preparations. Antibody R8 is electrophoretically heterogeneous, and is therefore a mixture of molecules. Presumably in such a mixture there are molecules which share nonprecipitating cross-specificity with the proband antibody. On the other hand, antibody R7 is a uniform antibody with a single major molecule species which has an electrophoretic mobility very different from that of the proband

antibody. In this case, because of the molecular uniformity, there are no molecules which share nonprecipitating cross-specificity with the proband antibody.

The conclusions to be drawn from the data in Table II are that allotypic combination, similarity of the binding sites, and the degree of antibody heterogeneity influence the extent of cross-specificity of antibodies.

Two types of experiments were performed to reveal whether the inhibitory excesses (I_{50} values) of preimmune IgG preparations are a true reflection of the proportion of molecules with cross-specificity for a proband antibody. In the first experiment a passive hemagglutination-inhibition system was used, which employed RBC coated with the proband antibody. In this inhibition system

TABLE III

*Noninhibitory Excesses of Various Preimmune IgG Preparations in Hemagglutination-Inhibition Test**

Source of IgG	Highest noninhibitory excess tested‡
R26–90 IgG	5400
Sibling 1 IgG	4080
Sibling 2 IgG	3950
Sibling 3 IgG	4940
R6 IgG	5260
R8 IgG	7640

*R26–90Ab coated to RBC, agglutinated with anti-R26–90Ab in 1:256 dilution.
‡ Excess over the lowest inhibitory proband antibody concentration (3.8 μg/ml).

only those molecules with complete cross-specificity for the proband antibody are inhibitory, and Kunkel has shown that this hemagglutination is much less susceptible to inhibition by IgG than is direct precipitation (19). In a second type of experiment, the amount of Group C antibodies in preimmune IgG was estimated, because only these antibodies are likely to possess cross-specificity for the proband antibody.

The results of the hemagglutination-inhibition test are listed in Table III. R26–90Ab was coated to RBC and the end point of agglutination by anti-R26–90Ab was obtained with a dilution of 1:256. This agglutination was readily inhibited by R26–90Ab at a concentration of 3.8 μg/ml. None of the preimmune IgG preparations, including R26–90 IgG, was inhibitory at a several thousand-fold excess over this concentration. These experiments indicate that in the case of the preimmune IgG of proband rabbit R26–90 the proportion of molecules with complete cross-specificity for R26–90Ab is less than 1 in 5000.

The proportion in preimmune IgG of molecules with anti-Group C carbohydrate activity was estimated by the modified Farr technique (21) which employed Group C carbohydrate labeled with ^{125}I. In order to determine the

binding capacity of Group C antibodies, Group C antisera were diluted to an antibody concentration of 30 μg/ml. 10–50 μl (0.3–1.5 μg Ab) of these diluted sera were then reacted with 100 ng of labeled carbohydrate, and the amount of antigen bound was between 50 and 70 ng of carbohydrate/1 μg of antibody. Similar experiments were performed with undiluted preimmune rabbit sera, including that of proband rabbit R26–90.

This serum contained 5 mg preimmune IgG/ml and 50 μl (250 μg IgG) bound 6 ng of labeled carbohydrate. If this is not a nonspecific effect, the preimmune serum of rabbit R26–90 contains approximately 2 μg of Group C antibody/ml, or 1 in 2500 IgG molecules are Group C antibody. Since it is unlikely that all of these "preimmune antibodies" possess cross-specificity for R26–90Ab, the proportion of such molecules is less than 1 in 2500.

Taken together, these data clearly indicate that the actual proportion of molecules with complete cross-specificity for a proband antibody in preimmune IgG is much smaller than is indicated by an I_{50} value. Nevertheless, the I_{50} values determined by quantitative inhibition of the direct precipitation of individually specific antisera revealed 30-fold differences in the nonprecipitating cross-specificity of preimmune IgG from proband rabbits, blood relatives, and nonrelated rabbits. These differences may be taken as a relative measurement of the proportion of molecules in an IgG preparation which has cross-specificity for a proband antibody.

DISCUSSION

Although there has been a remarkable advance in the knowledge of the chemical structure of the immunoglobulins, the genetic control of antibody structure has remained a controversial subject (27–29). This is due to the intrinsic obstacle that most conventionally induced antibodies, as well as the immunogloublins in nonimmune sera, are heterogeneous mixtures. Therefore, structural analysis has largely employed the homogeneous myeloma proteins. While amino acid sequences are now available on a considerable number, an evaluation of such sequence data has not provided a final solution to the genetic determination of immunoglobulin structure (30). The advantages of the rabbit antibodies to streptococcal carbohydrates as a tool for genetic studies are the molecular uniformity of these antibodies (4, 10, 12, 31) and the genetic transmission of this limitation in antibody heterogeneity (1). It has, therefore, been possible to demonstrate the inheritance of individual antigenic specificities of Group C antibodies from immunized rabbits of a selectively bred line.

IAS is a reflection of the unique amino acid sequence in the variable (V) region of an antibody molecule, and it is assumed that two antibody molecules which have identical IAS have identical amino acid sequences in this region. While comparative structural data are not yet available for antibodies to streptococcal carbohydrates with shared IAS, partial amino acid sequences are now well known for the L chains of several mouse myeloma proteins which

shared their idiotypes (32), and for the H and L chains of the IgG and IgM components of a human myeloma serum which had identical IAS (33). In each of these cases, shared antigenic individuality was reflected in identical V-region sequences.

Specificity for the same antigen appears to be required for two antibodies to share their IAS or idiotypes. Shared IAS was demonstrated among cold agglutinins of the IgM class (9) and between a mouse myeloma protein and an antibody from the same strain, both with specificity for the same antigen (34). Shared idiotypy was detected in IgG and IgM antibodies from the same animal (35). Furthermore, mouse myeloma proteins shared their idiotypes when they had specificity for the same antigen (32, 36). This suggests that the structure of the binding site participates in the composition of idiotypic and individually specific determinants (37).

Previous studies from this laboratory have shown that uniform antibodies to the streptococcal carbohydrates possess IAS (4), and that in a single rabbit, antibodies with identical IAS recur with repeated immunizations over an 18 month period (38), despite their absence during the interval between immunizations.

The present studies demonstrate that a given IAS can be preserved for three generations in a rabbit family. The Group C antibodies of a rabbit family have been screened for reactivity with individually specific antisera against the uniform antibodies of four proband rabbits. The IAS of 3 of these proband antibodies were also found in the antibodies in as many as 7 of the 42 members of the family, but were not found in Group C antibodies from 48 unrelated rabbits. Thus precipitating cross-specificity has been observed among Group C streptococcal antibodies only when they were obtained from related rabbits. In an earlier report, Braun and Krause described two Group C antibodies, derived from "random" rabbits, which had partically identical cross-specificities (4). However, subsequent careful evaluation of the source of these two rabbits could not exclude the possibility that they were littermates.

The cross-specificity of antibodies from related rabbits may be partial or complete, as was shown by immunodiffusion and radioprecipitin experiments. Examination by electrophoretic methods revealed that in the case of complete identity, the electrophoretic mobilities of the H and L chains from both antibodies are identical. In the case of partial identity, the electrophoretic mobilities of only one of the polypeptide chains were identical, whereas those of the other chain were different. This suggests that in some of these cases with partial identity, one of the polypeptide chains is common to both antibodies, whereas the other chain is different in both. Thus by the criterion of IAS, L and H chains can be inherited independently. Such an observation is not surprising because allotypic studies have shown that L and H chains are controlled by independent loci (39).

Radioprecipitin-inhibition studies with preimmune IgG have revealed that

the IAS of a proband antibody was shared by a small proportion of molecules in each rabbit IgG preparation tested. Preimmune IgG from the proband rabbit and from those relatives which developed antibodies with precipitating cross-specificity had the greatest inhibitory effect. Preimmune IgG from relatives which did not develop antibodies with precipitating cross-specificity were about one-half as effective. Preimmune IgG preparations from nonrelated rabbits which had the same group a allotype as the proband antibody had the same inhibitory effect as had IgG from those siblings which did not produce antibodies with precipitating cross-specificity. IgG preparations from rabbits with a different group a allotype were much less effective as inhibitors.

The I_{50} values measured by the radioprecipitin-inhibition test indicate that a 64-fold excess of preimmune IgG from proband rabbit R26–90 was required to achieve the same inhibition as was obtained with R26–90Ab. A similar excess was needed for the preimmune IgG from siblings 1 and 2, whereas a 120-fold excess was needed of preimmune IgG from siblings 3 and 4. However, when the same IgG preparations were used in passive hemagglutination-inhibition studies, which employed the proband antibody coated to RBC (19), several thousand-fold excesses of the same IgG preparations have not been inhibitory. This indicates that the actual proportion of molecules with complete cross-specificity is less than 1 in 5000. Furthermore, an estimation of the proportion of molecules with activity for the Group C streptococcal carbohydrate in preimmune IgG gave a value of 1 in 2500, but undoubtedly only a fraction of these molecules with Group C antibody activity possess the IAS of the proband antibody. While the difference in inhibitory capacities measured by precipitin inhibition and hemagglutination-inhibition experiments remains unexplained, these data clearly indicate that an I_{50} value is not a measurement for the actual proportion of molecules in preimmune IgG with complete cross-specificity for the proband antibody. Nevertheless, the 30-fold differences in the inhibitory capacities of preimmune IgG preparations from various rabbits as detected by the precipitin-inhibition test are an index of cross-specificity for the proband antibody. Thus, the I_{50} values of the preimmune IgG preparations are taken as a relative measure of the proportion of molecules with cross-specificity for a proband antibody.

It is an intriguing observation that the proportion of molecules with cross-specificity for a proband antibody is about equally large in the preimmune IgG of the proband rabbit and in the preimmune IgG of those family members which produced antibodies with precipitating cross-specificity. This proportion is only about one-half as large in the preimmune IgG of those family members which did not produce antibodies with the IAS of the proband antibody, and in the preimmune IgG of nonrelated rabbits which had the same group a allotype. Preimmune IgG of nonrelated rabbits with different group a allotypes contained a much smaller proportion of these molecules. Thus, it appears

that rabbits which responded with antibodies of this IAS have a relatively high concentration of molecules with cross-specificity in their serum before immunization. This suggests that selective breeding of rabbits which had responded with antibodies of restricted heterogeneity has yielded a population of rabbits with limited idiotypic variability.

The results obtained with both precipitating and nonprecipitating cross-specificity suggest that a given IAS is preferentially associated with a given group a allotype. It has been shown by the experiments in Table I that the guinea pig antisera used here do not recognize allotypic determinants. It is possible, however, that allotypic similarities enhance the inhibitory capacity of preimmune IgG by an interaction of both the individually specific and the allotypic determinants. To eliminate this possibility, studies are currently underway with idiotypic antisera prepared in allotypically matched rabbits. Preliminary results obtained with these antisera suggest that idiotypy is also an inherited character of Group C streptococcal antibodies.

It is concluded from these studies that repeated selective brother-sister mating has produced a rabbit population with a limited number of idiotypic alternatives for the response to Group C streptococcal carbohydrate. This could be explained by the assumption that V-genes are present as multiple pseudoalleles, and that inbreeding leads to idiotypic exclusion through the generation of homozygosity. The data suggest, that in the case of the rabbit H chain, the V-region pseudoalleles may be controlled by genes which are closely linked to those coding for the group a allotypic markers. In such a rabbit family with limited idiotypic variability, antigenic stimulation frequently selects antibodies with similar or identical IAS, and this IAS can be preserved over several generations. This observation argues against the occurrence of somatic mutation as the major mechanism for the generation of idiotypic variability (27). Rather it appears that each idiotype is an inherited gene product. Further studies will be directed toward comparison of the primary structure of antibodies with identical individual antigenic specificities.

SUMMARY

The inheritance of individual antigenic specificities (IAS) of rabbit antibodies to the Group C streptococcal carbohydrate was demonstrated in a selectively bred rabbit family. The IAS of the antibodies from 3 proband rabbits were also observed in the Group C antibodies in as many as 7 out of 42 related rabbits, but in none of the Group C antibodies from 48 unrelated rabbits. Immunodiffusion analyses and quantitative radioprecipitin experiments revealed that this cross-specificity may be either partial or complete.

Quantitative inhibition of the precipitin reaction between the proband antibody and its antiserum by preimmune IgG revealed 30-fold differences in the proportion of molecules with cross-specificity for the proband antibody. This

proportion is higher in the preimmune IgG of the proband rabbit and of those relatives which produced cross-precipitating antibodies than it is in the IgG of rabbits which had the same group a allotype, but did not produce cross-precipitating antibodies. The proportion is much lower in the IgG of rabbits with a group a allotype different from that of the proband antibody. These data suggest that serologically detected individual antigenic specificities are inherited markers of immunoglobulins.

The authors thank Dr. Richard M. Krause for his continuing support of this study. The generous gifts of antiserum and immunoglobulins from Dr. Charles W. Todd are gratefully acknowledged, and we thank both him and Dr. Henry G. Kunkel for helpful suggestions and comments. The enthusiastic participation of Mr. David Bernstein, Mr. Jay Greenblatt, and Miss Rochelle Seide made this work a special pleasure.

REFERENCES

1. Eichmann, K., D. G. Braun, and R. M. Krause. 1971. Influence of genetic factors on the magnitude and the heterogeneity of the antibody response in the rabbit. *J. Exp. Med.* **134**:48.
2. Kunkel, H. G. 1965. Myeloma proteins and antibodies. *Harvey Lect.* **59**:219.
3. Grey, H. M., M. Mannik, and H. G. Kunkel. 1965. Individual antigenic specificity of myeloma proteins. *J. Exp. Med.* **121**:561.
4. Braun, D. G., and R. M. Krause. 1968. The individual antigenic specificity of antibodies to streptococcal carbohydrates. *J. Exp. Med.* **128**:969.
5. Harboe, M., B. G. Solheim, and J. Deverill. 1969. Individual antigenic specificity of human macroglobulins. Localization of antigenic determinants and their use to demonstrate formation of hybrid molecules after reduction and reassociation. *J. Exp. Med.* **129**:1217.
6. Oudin, J., and M. Michel. 1963. Une nouvelle forme d'allotypie des globulines gamma de sérum de lapin aparement liée à la jonction et à la spécificité anticorps. *C.R.H. Acad. Sci. Ser. D.* **257**:805.
7. Kelus, A. S., and P. G. H. Gell. 1968. Immunologic analysis of rabbit antibody systems. *J. Exp. Med.* **127**:215.
8. Dougharty, H., J. E. Hopper, A. B. McDonald, and A. Nisonoff. 1969. Quantitative investigations of idotypic antibodies. I. Analysis of precipitating antibody populations. *J. Exp. Med.* **130**:1047.
9. Williams, R., H. G. Kunkel, and J. D. Capra. 1968. Antigenic specificities related to the cold agglutinin activity in γM macroglobulins. *Science* (*Washington*). **161**:379.
10. Krause, R. M. 1970. The search for antibodies with molecular uniformity. *Advan. Immunol.* **12**:1.
11. Braun, D. G., K. Eichmann, and R. M. Krause. 1969. Rabbit antibodies to streptococcal carbohydrates. Influence of primary and secondary immunization and of possible genetic factors on the antibody response. *J. Exp. Med.* **129**:809.
12. Eichmann, K., H. Lackland, L. Hood, and R. M. Krause. 1970. Induction of

rabbit antibody with molecular uniformity after immunization with Group C streptococci. *J. Exp. Med.* **131:**207.

13. Krause, R. M., and M. McCarty. 1962. Studies on the chemical structure of the streptococcal cell wall. II The composition of Group C cell walls and chemical basis for serological specificity of the carbohydrate moiety. *J. Exp. Med.* **115:**49.
14. Eichmann, K., and J. Greenblatt. 1971. Relationships between relative binding affinity and electrophoretic behavior of rabbit antibodies to streptococcal carbohydrates. *J. Exp. Med.* **133:**424.
15. Levy, H. B., and H. A. Sober. 1960. A simple chromatographic method for preparation of gammaglobulin. *Proc. Soc. Exp. Biol. Med.* **103:**250.
16. McFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. *Nature (London).* **182:**53.
17. Henney, C. S., and K. Ishisaka. 1969. A simplified procedure for the preparation of immunoglobulin class specific antisera. *J. Immunol.* **103:**56.
18. Porath, J., R. Axen, and S. Ernback. 1967. Chemical coupling of proteins to agarose. *Nature (London).* **215:**1491.
19. Kunkel, H. G. 1970. Individual antigenic specificity, cross-specificity and diversity of human antibodies. *Fed. Proc.* **29:**55.
20. Yvas, G. N., H. H. Fudenberg, H. M. Pretty, and E. R. Gold. 1968. A new rapid method for genetic typing of human immunoglobulins. *J. Immunol.* **100:**274.
21. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. *J. Infec. Dis.* **103:**329.
22. Reisfeld, R. A., and P. A. Small. 1960. Electrophoretic heterogeneity of polypeptide chains of specific antibodies. *Science (Washington).* **152:**1253.
23. Oudin, J. 1960. Allotypy of rabbit serum proteins. I. Immunochemical analysis leading to the individualization of seven main allotypes. *J. Exp. Med.* **112:**107.
24. Kindt, T. J., C. W. Todd, K. Eichmann, and R. M. Krause. 1970. Allotype exclusion in uniform rabbit antibody to streptococcal carbohydrates. *J. Exp. Med.* **131:**343.
25. Kindt, T. J., W. J. Mandy, and C. W. Todd. 1969. The action of cyanogen bromide on rabbit IgG molecules of allotypes A11 and A12. *Immunochemistry.* **7:**467.
26. Prahl, J. W., W. J. Mandy, G. S. David, H. W. Steward, and C. W. Todd. 1969. Participation of allotypic markers in rabbit immunoglobulin classes. *Protides Biol. Fluids Proc. Colloq. Bruges.* **17:**125.
27. Cohn, M. 1967. Antibody synthesis: the take home lesson. *Nobel Symp.* **3:**615.
28. Gally, J. A., and G. M. Edelman. 1970. Somatic translocation of antibody genes. *Nature (London).* **227:**341.
29. Hood, L., and D. W. Talmadge. 1970. Mechanism of antibody diversity; germ line basis for variability. *Science (Washington).* **168** 325.
30. Wu, T. T., and E. A. Kabat. 1970. An analysis of the sequences of the variable region of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J. Exp. Med.* **132:**211.
31. Hood, L., K. Eichmann, H. Lackland, R. M. Krause, and J. Ohms. 1970. Rabbit antibody light chains and gene evolution. *Nature (London).* **228:**1040.

32. Potter, M., R. Liebermann, L. Hood, and D. J. McKean. 1970. Structural and serological studies of six IgA myeloma proteins from Balb/c mice that bind phosphorylcholine. *Fed. Proc.* **29:**437.

33. Wang, A. C., K. Wilson, J. E. Hopper, H. H. Fudenberg, and A. Nisonoff. 1970. Evidence for control of synthesis of the variable regions of heavy chains of immunoglobulins G and M by the same gene. *Proc. Nat. Acad. Sci. U.S.A.* **66:**337.

34. Cohn, M., G. Notani, and S. A. Rice. 1969. Characterization of the antibody to the C-carbohydrate produced by a transplantable mouse plasmocytoma. *Immunochemistry.* **6:**111.

35. Oudin, J., and M. Michel. 1969. Idiotypy of rabbit antibodies. II. Comparison of idiotypy of various kinds of antibodies formed in the same rabbits against *Salmonella typhi*. *J. Exp. Med.* **130:**619.

36. Potter, M., and R. Liebermann. 1970. Common individual antigenic determinants in five of eight BALB/c IgA myeloma proteins that bind phosphoryl choline. *J. Exp. Med.* **132:**737.

37. Brient, B. W., and A. Nisonoff. 1970. Quantitative investigations of idiotypic antibodies. IV. Inhibition by specific haptens of the reaction of anti hapten antibody with its anti-idiotypic antibody. *J. Exp. Med.* **132:**951.

38. Eichmann, K., D. G. Braun, T. Feizi, and R. M. Krause. 1970. The emergence of antibodies with either identical or unrelated individual antigenic specificity during repeated immunizations with streptococcal vaccines. *J. Exp. Med.* **132:**1169.

39. Dubiski, S., J. Rapac, and Dubiska, A. 1962. Heredity of rabbit gammaglobulin iso-antigens. *Acta. Genet. Statist. Med.* **12:**136.