

## IDIOTYPIC SPECIFICITY OF RABBIT ANTIBODIES TO STREPTOCOCCAL GROUP POLYSACCHARIDES

BY DIETMAR G. BRAUN AND ANDREW S. KELUS

(From the Basel Institute for Immunology, Basel, Switzerland)

(Received for publication 15 August 1973)

Myeloma proteins are known to possess individual and idiotypic antigenic specificities (1-5), revealed by antisera raised within the same or a different species. Such specificities have also been demonstrated on antibody molecules directed against various antigens (6-10). They are located in the Fab fragment of the antibody (6, 8-10). Since the individual antigenic specificity (IAS)<sup>1</sup> and idiotypic specificity (ID), at least in the early experiments (2, 6-8), were shown to be strictly individual and not shared by other antibodies of the same specificity, it was assumed that IAS and ID reflected the individuality of this antibody in structural terms.

This original concept of the serological individuality of homogeneous immunoglobulins was curtailed by the observation that several members of a selectively bred rabbit family responded to the streptococcal Group C polysaccharide (C-CHO) with homogeneous antibodies of shared IAS (11) and the observation of cross-reacting IAS among several homogeneous antibodies of similar antigen specificities (3, 9, 12-14). Similar findings were reported for certain restricted antibodies raised in inbred mouse strains and mouse myeloma proteins with antibody activity (15-22). Even immunoglobulins with different specificities (23) were reported to share ID.

This paper deals with the ID specificities of homogeneous antibodies raised against streptococcal group polysaccharides in part-inbred rabbits. The data suggest that various degrees of cross-reactions may exist among Group A-variant antibodies in related but not in unrelated rabbits.

### *Materials and Methods*

*Rabbits.*—Rabbits for immunization with streptococcal vacaine originated from two sources (24). Details concerning the relationship of rabbits whose antisera and antibodies were employed to explore idiotypic cross-reactions are presented in the pedigree (Fig. 1). In addition antistreptococcal Group A-variant polysaccharide (anti-Av-CHO) antisera of 20 unrelated rabbits were used. Six Av-CHO antisera were raised in Dr. Krause's laboratory.

<sup>1</sup> *Abbreviations used in this paper:* Av-CHO, streptococcal Group A-variant polysaccharide; Av-CHO tyr <sup>131</sup>I, streptococcal Group A-variant polysaccharide tyraminated and <sup>131</sup>I labeled; C-CHO, streptococcal Group C polysaccharide; Fab fragment, fragment antigen binding; Fc fragment, fragment crystallizing; F(ab')<sub>2</sub> fragment, dimeric Fab fragment obtained by pepsin digestion; HRBC, human red blood cell; IAS, individual antigenic specificity; ID, idiotypic specificity; IEF, isoelectric focusing.

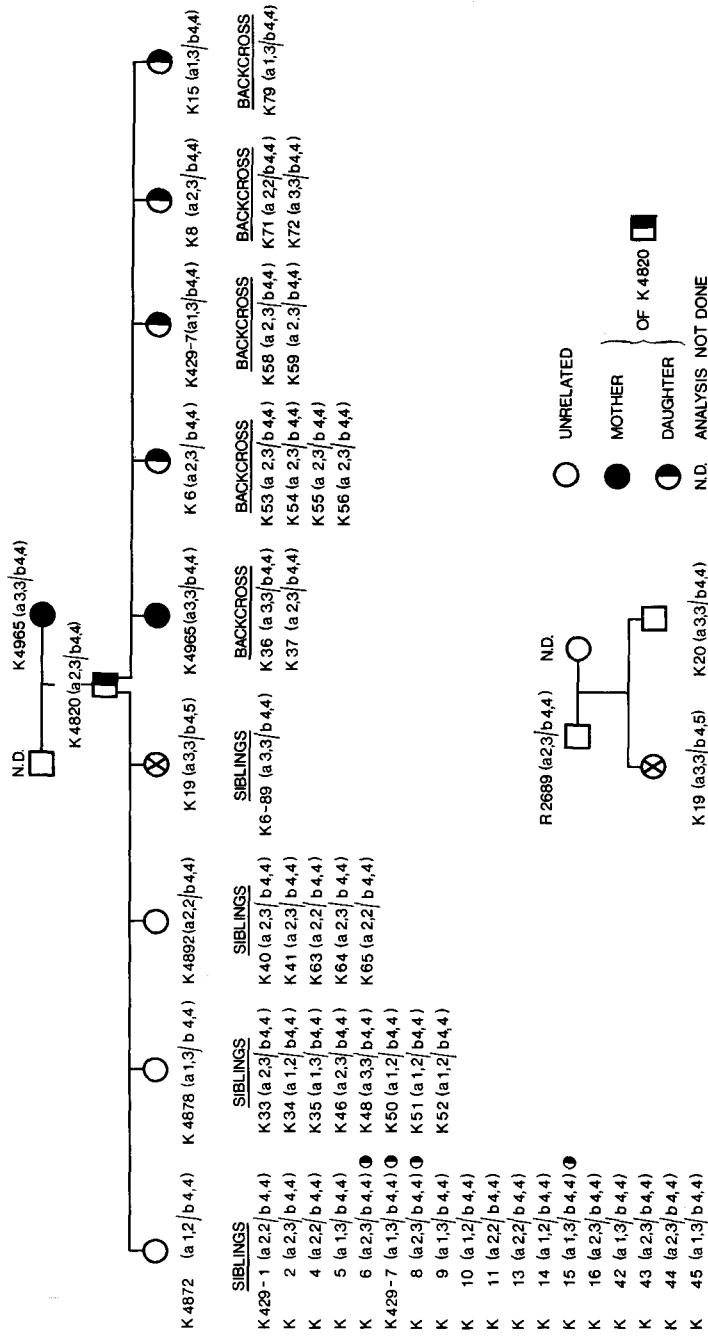


Fig. 1. Pedigree of the rabbit colony. Monoclonal high responder K4820 K8, K15) to yield backcross progeny. The allotypic specificities of the (24) was bred to four unrelated does (K4872, K4878, K4892, K19) to yield, preimmune  $\gamma$ -globulin are indicated for heavy chain a1, 2, 3 and light F<sub>1</sub> offspring, and to its mother (K4965) and four daughters (K6, K429-7 chain b4, 5, 6, 9 markers.

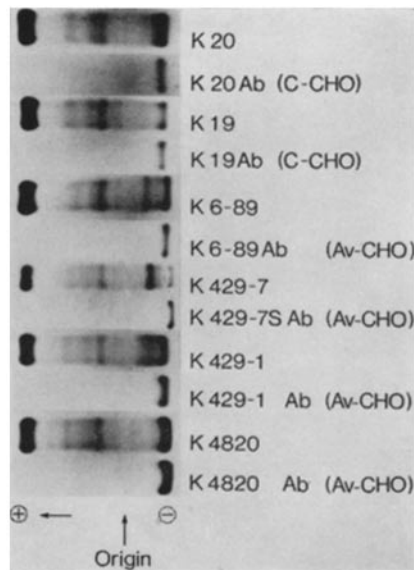


Fig. 2. Microzone electrophoresis patterns of whole immune sera and corresponding isolated antibodies used for the production of anti-idiotypic antisera. The serological specificity for the streptococcal polysaccharides is indicated in parentheses.

Among them was R2250 which besides two very distinct anti-Av-CHO antibodies (9) was reported to contain 19S anti-Ig antibodies in a titer of 1:40,000 (25). Furthermore, purified restricted antipneumococcal Type III antibodies from two rabbits of a third colony were kindly supplied by Dr. J.-C. Jaton, The Basel Institute for Immunology.

Rabbit antibodies K4820, K429-1, K429-7s, K6-89, K19, and K20 were chosen as proband antibodies (Fig. 2). Rabbits which were allotypically matched but unrelated to the above were selected for the production of anti-idiotypic antibodies.

*Streptococcal Vaccines and Immunization.*—The streptococcal vaccines used for intravenous immunization (24) included those from strains of serological Group A-variant (A486 var.M-), and C (C74) obtained from Dr. Rebecca C. Lancefield, The Rockefeller University, New York.

*Streptococcal Group Polysaccharides.*—Isolation of the group polysaccharides was accomplished by the hot formamide extraction of Group A-variant and C streptococcal cell walls (26).

*Electrophoretic Methods.*—Immune sera were screened for the presence of restricted antibody bands by microzone electrophoresis (27). The degree of restriction was determined further by analytical isoelectric focusing in polyacrylamide gels (24, 28).

*Isolation of Antibody Components.*—Preparative electrophoresis and re-electrophoresis in 0.5% agarose (SeaKem®, Marine Colloids Inc., Springfield, N. J.) were employed for the isolation of streptococcal group-specific antibodies (9).

*Preparation of Anti-Idiotypic Antisera.*—The six restricted antibodies (Fig. 2) were injected into allotypically matched rabbits. For immunization, 1 mg of isolated antibody was allowed to agglutinate an excess of washed homologous streptococcal cells to give 0.5 ml suspension. When a heavy agglutinate had formed this was emulsified with 0.5 ml of complete Freund's adjuvant and injected intramuscularly. Generally, recipients produced precipitating anti-ID antibodies after six injections.

*Direct Precipitation.*—Double diffusion in 1.5% agarose gels (pH 7.2) was used to assay for the presence of ID precipitation and for cross-specificity in antibody fractions and whole antisera from kindreds and unrelated rabbits, all heat inactivated.

*Hemagglutination and Hemagglutination Inhibition.*—O Rh human red blood cells (HRBC) were coated with isolated antibodies by the chromic chloride technique (30). For hemagglutination inhibition only homologous ID coats were used. All antisera were heat inactivated and absorbed with HRBC.

*Sheep Antiserum to Rabbit Fragment Crystallizing (Fc) Fragments.*—A sheep antiserum to purified rabbit IgG was absorbed with 1 mg/ml of pooled rabbit IgG F(ab')<sub>2</sub> fragments prepared by pepsin digestion (31). The absorbed antiserum failed to react with rabbit IgG F(ab')<sub>2</sub> fragment in passive hemagglutination, while reaction with rabbit IgG-coated red cells showed an endpoint of hemagglutination of 1:42,000.

*Preparation of Fab Fragments and Labeling with <sup>125</sup>I.*—Fab fragments were prepared by papain digestion and carboxymethyl-cellulose chromatography (32) of 10 mg each of K4820 Ab and K429-1 Ab; 100–200 μg of Fab were labeled with <sup>125</sup>I by the chloramine-T method (33). The specific activity of the labeled Fab fragments was 5.6 × 10<sup>4</sup> cpm/μg for K4820 Ab Fab and 1.2 × 10<sup>4</sup> cpm/μg for K429-1 Ab Fab.

*Precipitation and Precipitation Inhibition.*—From 72–85% of the <sup>125</sup>I-labeled Fab fragments could be precipitated by homologous anti-idiotypic antisera. Inhibition studies with cold restricted anti-Av-CHO antibody fractions were carried out at 65–70% precipitability of the homologous Fab <sup>125</sup>I with its antiserum. This point was selected from the ascending part of the precipitation curve. A total of 1.1–1.2 × 10<sup>4</sup> cpm was added to each test. All tests were carried out in 1% bovine serum albumin made in 0.9% NaCl, 0.015 M sodium phosphate buffer, pH 7.7. In detail, cold inhibitor and anti-idiotypic antibody were mixed first in the presence of 50 μg of rabbit IgG (Pentex Biochemical, Kankakee, Ill.), and incubated for 60 min at 37°C. <sup>125</sup>I-labeled homologous Fab fragment was then added and the mixture incubated for 30 min at 37°C, after which time 500 μl of a sheep antirabbit Fc antiserum was added and incubation proceeded for another 30 min. The tubes were then left at 4°C overnight, spun in a refrigerated centrifuge at 5,000 rpm for 20 min, and both supernatants and precipitate fractions counted in a Packard Liquid Gamma Scintillation Counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

## RESULTS

A high degree of uniformity of the antibodies used here as antigens (ID) for the induction of anti-ID antisera was apparent from independent observations, e.g., their isoelectric focusing patterns and N-terminal light chain sequence (29, 34). Limited sequence information on the heavy chains of K4820, K19, and K20 antibodies supported this interpretation (35). When tested for their allotypic specificities (36), all antibodies were found homozygous for both light and heavy chain markers (Table I).

*Specificity of the Anti-Idiotypic Antisera.*—The idiotypic antisera were tested for specificity by double immunodiffusion and passive hemagglutination. The hemagglutination titers varied in homologous idio-anti-idio systems between 1:1,280 and 1:20,480 (Table I). No reaction was obtained with coats of pooled rabbit IgG (Pentex) on HRBC. Immunodiffusion studies employing the homologous preimmune sera failed to precipitate with the homologous anti-idiotypic antisera; so did rabbit IgG of the same allotype. These data are

TABLE I  
*Allotypic Specificities of Isolated Homogeneous Antibodies with ID. Passive  
 Hemagglutination Titers of Anti-Idiotypic Antisera  
 against Homologous Idiotypes*

Antibody	Allotype		Specificity	Anti-idiotypic antisera	Hemagglutinin titers
	H-chain	L-chain			
K4820 Ab	a2,2	b4,4	Strept. Av-CHO	BS 194	1:1280
K429-1 Ab	a2,2	b4,4	Strept. Av-CHO	BS 976	1:20480
K429-7s Ab	a1,1	b4,4	Strept. Av-CHO	BS 288	1:10240
K6-89 Ab	a3,3	b4,4	Strept. Av-CHO	BS 952	1:20480
K19 Ab	a3,3	b4,4	Strept. C-CHO	BS 264	1:1280
K20 Ab	a3,3	b4,4	Strept. C-CHO	BS 464	1:5120

consistent with the idea that all anti-idiotypic antisera were specifically directed against variable region determinants of the idiotypic antibodies.

*Cross-Reactions with ID Antisera.*—Analyses in double immunodiffusion yielded cross-reactive specificities of 5 ID antisera with 42–63% of the immune sera from rabbits listed in the pedigree (Fig. 1). These cross-precipitations appeared to be independent of the genetic background of the individual animal. Antiserum BS288 reacted only with the inducing K429-7s Ab.

As this percentage of cross-reactivity was unusually high, rheumatoid factor was suspected to mimic idiotypic cross-precipitation. Rheumatoid factor is known to occur in streptococcal group-specific antisera (25). For further exploration of this aspect and for subsequent experiments using the binding inhibition technique employing monomeric <sup>125</sup>I-labeled Fab, we restricted our work to two anti-ID antisera (BS194: anti-K4820 Ab; and BS976: anti-K429-1 Ab).

Immunodiffusion analyses employing the Group A-variant antisera of 20 unrelated rabbits revealed precipitation of three of them with BS194 and of the same three and two additional antisera with BS976. None of the A-variant hyperimmune antisera showed precipitating activity against pooled rabbit IgG (1 mg/ml).

All subsequent tests were therefore performed under the assumption that the precipitating cross-reactions observed between streptococcal Group A-variant antisera and two anti-ID antisera were in part caused by rheumatoid factors which precipitated with immune complexes in the anti-ID antisera (25). The aim was to absorb out the cross-reactive principle. This was successfully achieved for all cross-reactive Group A-variant antisera (100  $\mu$ l) if mixed with 10<sup>9</sup> pneumococcal Type III cells to which subsequently 100  $\mu$ l of pooled heterogeneous Type VIII pneumococcal antiserum were added for agglutination. Before this, it was verified by a slide agglutination test that these A-variant antisera did not agglutinate pneumococcal Type III cells. After this treatment the precipitin reaction with the inducing isolated idiotypes was unim-

paired. Also, after a similar treatment of these antisera, the cross-reactions seen with the streptococcal group antisera had disappeared with two exceptions: BS194 still reacted in a very faint precipitate with K16 antiserum, and BS976 with K35 antiserum. In both instances spur formation of the homologous over the heterologous reaction was less pronounced than the unabsorbed group Av-CHO antisera.

It was our interpretation at this point that with two exceptions (K16 and K35) the observed absorbable cross-precipitations were caused by the presence of anti-immunoglobulins; these were trapped as the Type III-specific antigen-antibody reaction occurred and thereby were removed to interfere in immunodiffusion analyses. Because the cross-reactions were so easily absorbable in the presence of an unrelated antigen-antibody reaction and because they were expected to bear also on the results obtained by direct passive hemagglutination, further experiments were limited to isolated antibody fractions of the immune sera within the pedigree (Fig. 1).

In an independent series of experiments, it was shown that several isolated monoclonal antibodies occurring in individual antisera of the progeny described here not only focused at a different isoelectric point (34) as compared to K4820 Ab, but in addition did not significantly cross-react with the anti-idiotypic antisera raised against K4820 Ab and K429-1 Ab, respectively. Subsequently, only those fractions were analyzed for idiotypic cross-reactivity with parent antibody K4820 and sibling antibody K429-1 which had a similar electrophoretic mobility and banding properties in isoelectric focusing (IEF) to that of K4820 Ab. Not a single antiserum of the 20 unrelated rabbits showed IEF patterns of their Av-CHO specific antibodies similar to either K4820 Ab or K429-1 Ab.

*Isoelectric Focusing Spectra of Parent K4820 Ab and Progeny Antibodies.*— Fig. 4 shows the IEF pattern of K4820 serum. It appears that two clones may be present in this preparation, called clone *a* and clone *b*, of which clone *b* accounted for approximately 90% of the isolated antibody. When progeny antisera were tested for the presence of similar clones, their identification was not possible unless three to four times the amount of antiserum was subjected to IEF. A typical example is given in Fig. 3. Both parent K4820 and offspring K53 (a backcross progeny) antisera were run along each other. It appears that the K53 and K4820 antisera contain antibodies of a very similar pattern (pH 7–8). In addition, K53 possesses a predominant monoclonal antibody at pH 6.1–6.3.

In order to screen the antisera from offspring for the presence of similar antibodies, 5-ml serum samples of 25 F<sub>1</sub> and 11 backcross offspring were subjected to preparative agarose block electrophoresis to purify crudely the IgG which migrated electrophoretically like K4820 Ab. The antibodies isolated in this region bound tyraminated and <sup>125</sup>I-labeled Av-CHO (Av-CHO tyr <sup>125</sup>I) specifically as was evident from the autoradiograms of IEF gels (24, 37). Such

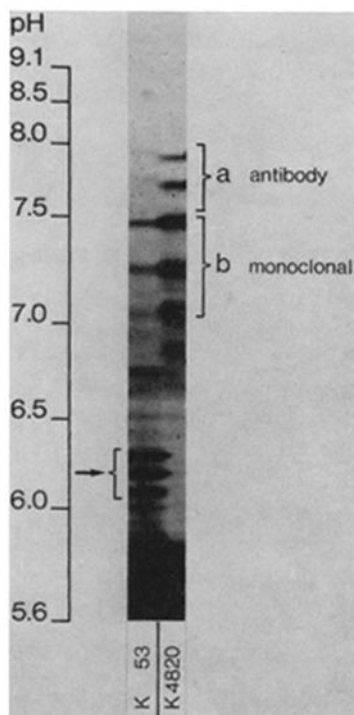


Fig. 3. IEF patterns of antisera K4820 and K53. An equivalent of the predominant clone of K4820 antiserum is also expressed in K53 antiserum; its predominant monoclonal antibody focuses at pH 6.1–6.3.

analysis revealed that 17 antisera contained antibodies similar to K4820 clonal products a and b, three antisera contained antibodies similar to clonal antibody a, and 13 antisera contained antibodies similar to clonal antibody b; some of these are shown in Fig. 4. Three antisera were devoid of antibodies similar to either a or b.

These crudely purified antibodies were analyzed for idiotypic cross-reaction by the radiobinding inhibition test for the estimation of degrees of similarities, and the hemagglutination inhibition assay to test for identity of highly cross-reactive antibodies.

*Radiobinding Inhibition Analyses.*—Binding inhibition analyses (38) were performed with two systems: K4820 Ab Fab  $^{125}\text{I}$  and the homologous anti-ID antiserum, and K429-1 Ab Fab  $^{125}\text{I}$  and its anti-ID antiserum. The specific idiotype antiidiotype binding of this system is unlikely to be affected by the presence of anti-IgGs when whole immune sera are used as inhibitors, because rheumatoid factor recognizes determinants only of the Fc portion (39) under conditions of coprecipitation (25); in this case of kindred samples it was further excluded because purified IgG fractions mainly consisting of Av-CHO-

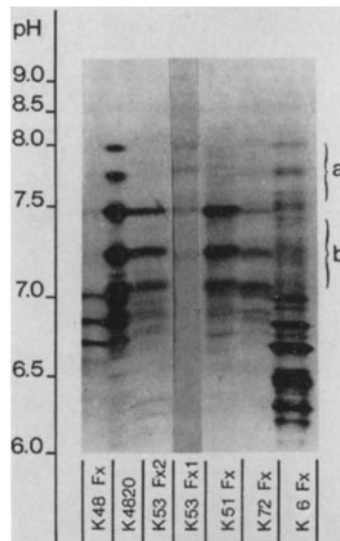


Fig. 4. IEF patterns of K4820 antiserum and of IgG fractions (Fx) of offspring antisera. All of the stained bands contained, in corresponding positions to clones a and/or b, antibodies with specific binding activity for the Av-CHO tyr  $^{131}\text{I}$ : K48 Fx (a), K53 Fx 1 (a + b), K53 Fx 2 (b), K51 Fx (b), K72 (a + b), K6 (a + b). K53 Fx 1 track was separately exposed because of its very faint staining. The additional strongly stained bands, e.g. K48 Fx and K6 Fx in the range of pH 6.0-7.0, also consisted of Av-CHO-specific antibody.

specific antibodies were used to test for binding inhibition. In the homologous reaction, 82% of K4820 Ab Fab  $^{125}\text{I}$  and 73% of K429-1 Ab Fab  $^{125}\text{I}$  were precipitable. In binding inhibition analyses with cold homologous idiotype, 91 and 96% inhibition was achieved with 20-fold (10  $\mu\text{g}$ ) and 16-fold (80  $\mu\text{g}$ ) excess, respectively, and appropriate inhibition curves obtained (Fig. 5, 6). Duplicate determinations with cold inhibitor were carried out at 20-fold excess in the K4820 Ab system, and at 16-fold excess in the K429-1 Ab system. The high excess of heterologous Ig was chosen to cover even minor inhibitory components. The inhibition test with pooled IgG as negative control was included (Fig. 5, 6).

Av-CHO-specific antibody fractions from 36 related rabbits were tested for their capacity to inhibit binding of homologous Fab  $^{125}\text{I}$ . Binding of K4820 Ab Fab  $^{125}\text{I}$  by its anti-ID antibody was only efficiently inhibited by one offspring (K16) antibody (Fig. 5). This inhibition amounted to 72% of the homologous reaction (Fig. 7). K16 antibody of the same heavy- and light-chain allotypes of K4820 Ab also showed a weak but definitive precipitin reaction with BS194 on immunodiffusion analysis. Another antibody produced by a backcross offspring (K36) achieved 42% inhibition. This antibody, similar to four F<sub>1</sub> offspring and one additional backcross antibody, which inhibited the ID anti-ID reaction between 36-40% (Fig. 7), was unreactive with BS194 antiserum in



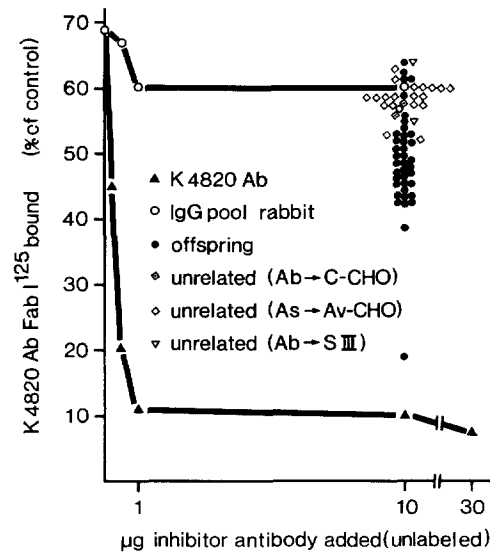


Fig. 5. Precipitation inhibition analyses performed with K4820 Ab Fab  $^{125}\text{I}$  and its homologous anti-idiotypic antiserum (BS194). Triangles, circles, dots, squares, and dotted squares represent the mean value of a duplicate or triplicate determination with the respective cold inhibitor fraction.

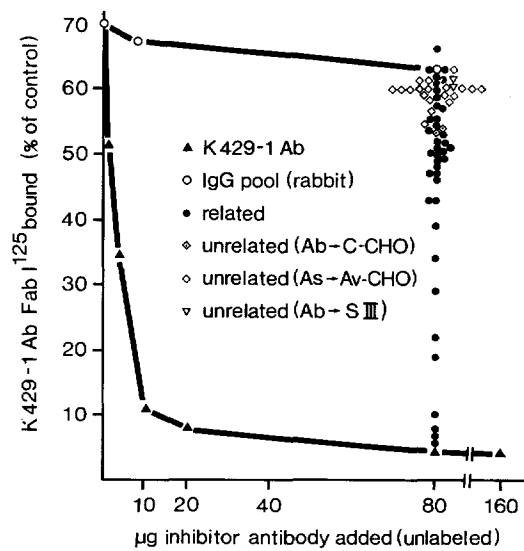


Fig. 6. Precipitation inhibition analyses performed with K429-1 Ab Fab  $^{125}\text{I}$  and its homologous anti-idiotypic antiserum (BS976). Triangles, circles, dots, squares, and dotted squares represent the mean value of a duplicate or triplicate determination with the respective cold inhibitor fraction. These were the same as in Fig. 5.

immunodiffusion analyses. About 64% of antibodies from related rabbits, however, inhibited the homologous reaction less than 30% (Table II).

Anti-Av-CHO antisera of 20 unrelated rabbits were added in amounts which accounted for 10  $\mu\text{g}$  of Av-CHO antibodies when tested in the K4820 ID system (Fig. 5) and for 80  $\mu\text{g}$  of Av-CHO antibodies in the K429-1 ID system

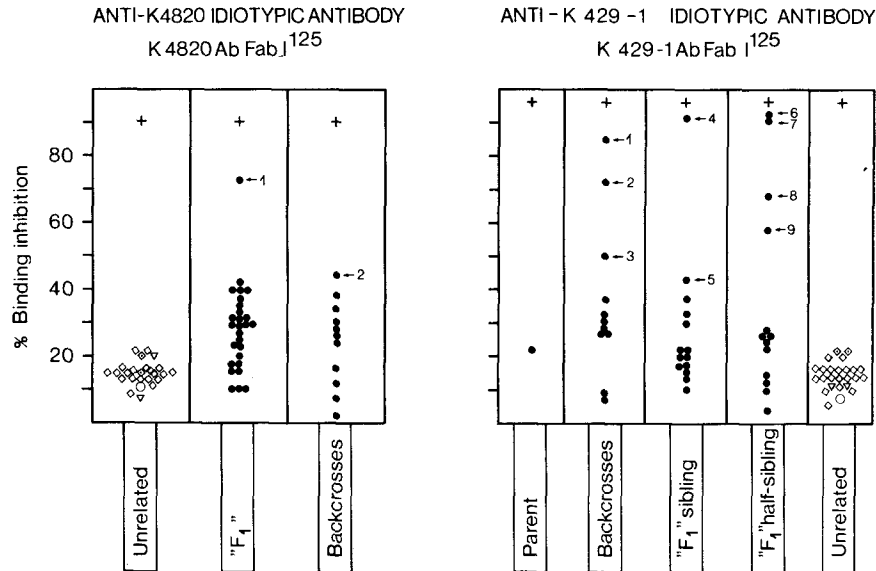


Fig. 7. Binding inhibition capacity of purified antibodies, IgG-fractions or whole antisera shown in Fig. 6 and 7 by taking 70% precipitability of homologous Fab  $^{125}\text{I}$  as 100%. Degree of inhibition by the homologous cold inhibitor is indicated by +. All other symbols used here are the same as in Fig. 6 and 7. Additional symbols: *Anti-K4820 ID system*:  $\bullet \leftarrow 1 \triangle K16$ ;  $2 \triangle K36$ ; *Anti-K429-1 ID system*:  $\bullet \leftarrow 1 \triangle K54$ ;  $2 \triangle K58$ ;  $3 \triangle K72$ ;  $4 \triangle K16$ ;  $5 \triangle K429-7s$  Ab;  $6 \triangle K35$ ;  $7 \triangle K51$ ;  $8 \triangle K6-89$  Ab;  $9 \triangle K41$ .

TABLE II

*Percent Differences of Binding Inhibition of K4820 and K429-1 ID Anti-ID Systems Observed with Specific Antibodies of Related and Antisera of Unrelated Rabbits*

Percent binding inhibition	BS194 $\rightarrow$ 4820 Fab $^{125}\text{I}$				BS976 $\rightarrow$ 429-1 Fab $^{125}\text{I}$			
		%		%		%		%
>50	1*	2.5	0†	0	7*	18.0	0†	0
31-50	13*	33.3	0†	0	6*	15.4	0†	0
21-30	13*	33.3	3†	12.5	13*	33.3	2†	8.3
<20	12*	30.9	21†	87.5	13*	33.3	22†	91.7

\* Number of antibodies of related rabbits (39 tested).

† Number of antisera or antibodies of unrelated rabbits (24 tested).

(Fig. 6). In addition, two purified C-CHO-specific antibodies (K19 and K20) and two purified pneumococcal Type III-specific antibodies were added as inhibitors to both systems. These rabbits were unrelated to those in the pedigree (Fig. 1) with the exception of K6-89 (Fig. 1). Only one Type III-specific antibody inhibited the K4820 ID system up to 21%. K19 and K20 antibodies achieved 22% inhibition in the K429-1 ID system. The great majority of Av-CHO antisera from unrelated rabbits inhibited specific binding in both ID systems between 10 and 20% and thereby somewhat better than Pentex pooled rabbit IgG (Fig. 7).

Idiotypically similar specificities among A-variant-specific antibodies of related rabbits were more readily detected if the proband idio type was produced by the F<sub>1</sub> generation offspring K429-1 rather than by the parent K4820 (Fig. 6, 7; Table II) although differences of the IEF pattern of K429-1 Ab and K4820 Ab were clearly demonstrable. Not a single pattern of the antibodies used for comparison had the same degree of similarity as was observed for many of them with K4820 Ab. Thus, this finding emphasizes that similar or identical isoelectric points of antibodies is not necessarily indicative for shared idiotypes.

Four antibody fractions (K35, K51, K16 and K54) showed remarkable ID similarities with K429-1 Ab (Fig. 7). Among these was K16 antibody, which also significantly inhibited the reaction of K4820 Ab and its anti-ID antiserum. Three additional antibody fractions (K58, K72, K41) and two purified anti-A-variant antibodies (K429-7s Ab, K6-89 Ab) showed greater than 40% inhibition. Three of these antibodies (K35, K429-7s, K6-89) did not share the heavy chain allotype a<sub>2</sub> with K429-1 Ab, while their light chains were also of the b<sub>4</sub> specificity. Four antibodies of related rabbits inhibited K429-1 Fab binding between 30 and 40% while the remaining 26 antibody fractions were less than 30% inhibitory in this system. In comparison to these results, the inhibitory capacity of antibodies of unrelated rabbits was much lower and comparable for both ID systems (Table II).

Such a finding of various degrees of serological similarities strongly suggests that ID specificities of monoclonal antibodies may be composed of more than one determinant. The degree of similarity should therefore be a function of the number of shared determinants. Most antistreptococcal Group antibodies were somewhat better inhibitors than pooled rabbit IgG (Pentex) on an equal molar basis. This may be a reflection of certain structural features shared by anti-polysaccharide antibodies of the same specificity even when produced in unrelated rabbits.

*Hemagglutination Inhibition Analysis.*—Hemagglutination inhibition analysis employed homologous idio type coat on HRBC and free heterologous inhibitor. In an attempt to test for idiotypic identity it was only performed with antibody fractions which by IEF and radiobinding inhibition analysis had shown a considerable degree of similarity to antibodies K4820 and K429-1. The results may be briefly summarized as follows: while a high degree of inhibition was

found for both homologous idio type anti-idio type systems K4820 Ab and K429-1 Ab, only insignificant inhibition (6-10 dilution step difference) was obtained with heterologous antibody fractions if equal amounts of inhibitor antibody were added starting with a dilution of 0.05 mg/ml. These data would imply that although significant similarities do exist between Av-CHO specific antibodies induced in related rabbits, structural identity could not be established.

Hemagglutination inhibition by homologous antibodies in the presence of excess pooled rabbit IgG allowed testing of the minimal concentration of the homologous idio type required as inhibitor of the idio type anti-idio type system (3). The dilutions of the three idio types investigated were made in the presence of 10 mg/ml of pooled rabbit IgG. The results were summarized in Table III. For example, K429-1 idio type was still inhibiting the reaction when present at

TABLE III  
*Hemagglutination Inhibition in the Presence of 10 mg Pooled Rabbit IgG/ml*

Ab preparation	Anti-idiotypic antiserum	Last inhibitory dilution	Minimal no. of idiotypic molecules per molecules IgG
K4820 (1 mg/ml)	Homologous BS 194 1:1280	1:1024	1:10000
K429-1 (1 mg/ml)	Homologous BS 976 1:10240	1:64000	1:640000
K429-7s (1mg/ml)	Homologous BS 288 1:5120	1:4000	1:40000

a concentration of only  $6.4 \times 10^{-4}$  mg/ml at an anti-idiotypic antiserum dilution of 1:10,000. Thus, less than 1 molecule of K429-1 Ab molecule per  $6.4 \times 10^5$  IgG molecules was sufficient for inhibition. These data may indicate that the rabbit has a potential of at least  $6 \times 10^5$  different antibody variable regions. The finding furthermore suggests that antibodies against the streptococcal Group A-variant antigen may be taken from a rare subpopulation.

*Persistence of Idiotypic Antibodies.*—In an earlier experimental series, the restimulation of monoclonal antibodies specific for the streptococcal Groups A-CHO and C-CHO was magnified during a secondary course of immunization (40). This aspect was reinvestigated for 15 homogeneous Group Av-CHO-specific antibodies by analytical IEF and for K429-1 Ab, K429-7s Ab, and K6-89 Ab by both IEF and use of anti-idiotypic antisera. Persistence of clearly distinct monoclonal antibodies during 1° and 2° immunization was found in all cases. In the three samples studied for the occurrence of identical idiotypes after a 1° and a 2° immunization 6 mo later no qualitative change of the early and late occurring idiotypes was noted. Their IEF patterns were identical, as shown in Fig. 8.

This observation supports the view that if a clone of antibody-forming cells is once stimulated to significant numbers or even to clonal predominance it

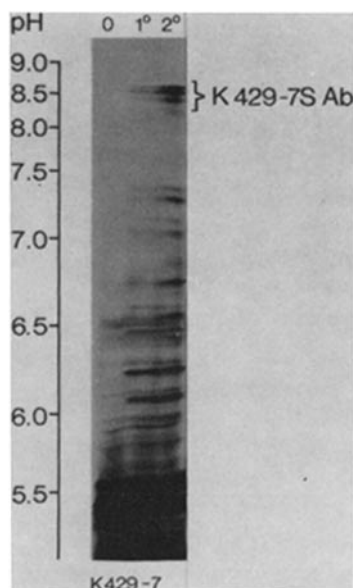


Fig. 8. IEF patterns of various sera obtained from K429-7: preimmune sera (0), primary (1°), and secondary (2°) immune sera. Persistence of the K429-7s Ab is particularly striking. Monoclonal antibodies focusing at various other pIs also persisted, e.g., pH 5.9–6.2, 6.5–6.7, and 7.1–7.4.

will be restimulated in the later history of the animal during a similar antigen contact implying its persistence.

#### DISCUSSION

One of the promising approaches to problems of antibody variability has been the stimulation of molecularly similar antibodies of identical antigen specificity in rabbits selectively bred for the trait of homogeneous high response to the streptococcal Groups A, A-variant, and C polysaccharides (11, 24, 27, 46).

Although individual antigenic specificity (IAS) of the Group C antibodies produced by closely related rabbits appears to follow some heritable pattern, the nature of the determinants involved has remained obscure (11). It is conceivable that shared IAS reflects an ID broader than that of the proband antibody. Thus, a more confined degree of specificity possibly residing in or close to the combining site of the idio- type is expected if anti-ID antibodies are raised homologously (41). For the idiotypes used in this study, evidence is available that some of the anti-idiotypic antibodies do compete with the isolated soluble polysaccharide antigen for binding.<sup>2</sup>

Double-diffusion analyses in agarose gel revealed a high frequency of cross-reactive specificities when whole antisera were tested due to the interference of rheumatoid factor. Recently, Kindt et al. reached a similar conclusion (42). The presence of anti-

<sup>2</sup> Pink, J. R. L., D. G. Braun, and A. S. Kelus. Unpublished data.

IgG antibodies in streptococcal Group antisera was emphasized by Bokisch et al. (25); earlier they were also reported for antisera against gram-negative bacteria (39) and a variety of antigens (43-45).

Within the present studies, emphasis was put on binding inhibition assays (38). With this technique, extensive idio-type cross-reactions were demonstrable between streptococcal Group Av-CHO antibodies of related but not of unrelated rabbits. Such results seem to contrast strongly with those reported by Kelus and Gell (8), but they confirm results obtained in other cross-reacting idiotypic systems (9, 11-23).

Substantial idiotypic cross-reactions reiterate the question about the nature and the location of the idiotypic determinant. The data obtained in this study suggest that an idio-type is composed of several determinants. Therefore, the observation of idiotypic cross-reactivity supports the assumption that cross-reactive idiotypes share only part of, but not the entire variable region of heavy and light chains. This interpretation correlates with the finding in the mouse system where the idio-type can include several antibodies with different sequences (22).

There is accumulating evidence that selective inbreeding of rabbits for homogeneous antibodies to bacterial polysaccharides narrows the spectrum of alternative antibodies (11, 24, 42). For example, the high frequency of antibodies with similar charges may result from the inheritance of basic structural genes for heavy and light chain variable regions (24). This electrophoretically similar or identical behavior could mask silent amino acid substitutions expressed in uncharged amino acids. High frequencies of an electrophoretically silent polymorphism were recently described for primate hemoglobins (47). Amino acid substitutions in antibodies involving charged amino acids may influence selective advantages; a selected active molecule may therefore be preferentially expressed, once achieved (24). Limited information on antibody light chain N-terminals appears to support this view; the N-terminal 20-30 amino acids of homogeneous antibody light chains seem to reflect the breeding relationship of these rabbits (29).

A complex situation was recently emphasized by an observation of Kindt et al. who reported idiotypic cross-reactions between antibodies of a single rabbit (48). The two antibodies, however, differed by their heavy chain allotype and IEF pattern. This may correlate with our results which suggest that similar but not identical idiotypic determinants appear to be present in antibodies from different but closely related individuals, although their heavy chain allotypes differed.

In genetic terms, the present data indicate the inheritance of idiotypic markers. If single genes for light and heavy chain variable regions of antibodies were transmitted as germ-line genes and if this information was transcribed into identical sequences within closely related rabbits, identical idiotypic molecules should be detectable. Instead, only partially shared idiotypic specificities are observed. This rather complicated pattern of inheritance of idiotypic similarities could be explained by the follow-

ing interpretation: variable region genes for the expression of antibodies of identical antigen specificities are inherited to the extent that they may be indistinguishable by their charge properties (24), and that their light chains may belong to the same subgroup (29). Thus, the relatively constant sequence portions within the variable regions of heavy and light chains would be conserved. This basic information transfer may then be modified by superimposed somatic mechanisms, e.g., episomal gene transfer specific for the hypervariable regions (48, 49). An alternative possibility could be offered by the following hypothesis. It suggests that variable region genes coding for antibodies with defined specificities for groups of antigens, e.g. polysaccharide determinants, are clustered at a defined locus of the chromosome separately for light and heavy chains. Maternal and paternal DNA strands carrying similar information should be in homologous positions which may favor cross-over and double cross-overs to occur in synaptonemal complexes (50). It is suggested that the three hypervariable regions of light and heavy chains are the locations to which such cross-over events are restricted. This model could explain why, on the one hand, constant amino acid sequence sections are maintained, and why antibodies of different heavy chain allotypes may share a similar or the same idiotype.

Clarification of the complex pattern of idiotypic similarities may emerge from the determination of the amino acid sequence of selected antibody molecules raised in related and unrelated rabbits.

#### SUMMARY

Anti-idiotypic antisera against six restricted rabbit streptococcal group specific antibodies have been raised in rabbits matched for allotypes. All these antisera reacted specifically with their homologous idiotypes on double-diffusion tests in agarose gel. In addition, they showed a high incidence of cross-specificities with group-specific hyperimmune sera induced in both closely related and unrelated individuals. These precipitating cross-specificities could be explained for two systems by the interference of rheumatoid factor.

Two idiotypic antibody systems have been analyzed in detail; these were restricted antibodies produced in a father and in one of his offspring. The methods employed included binding inhibition of radio-labeled homologous Fab fragments and hemagglutination inhibition with homologous idiotypic coat. The data demonstrated that only related rabbits produced, besides non-cross-reacting antibodies, idiotypically similar antibodies raised to the same antigen. About one-third of the cross-reactive idiotypes showed binding inhibition between 31 and 92%. Inhibition of binding above 50% in the paternal idiotypic system was only achieved by one offspring antibody whereas the F<sub>1</sub> progeny idiotypic system was inhibited to this extent by seven antibodies of related rabbits. In contrast, 87.5% and 91.7% of antibodies of unrelated rabbits were less than 20% inhibitory.

Within this study two idiotypically identical antibodies have not been found. This implies that A-variant-specific antibodies of related rabbits which produced antipolysaccharide antibodies were structurally different. Cross-reaction,

even if greater than 90% by binding inhibition, appears to involve only part and not all of the variable regions.

The competent technical assistance of Miss K. Hild, Mrs. E. Hillebrand, Miss D. Wiegardt, and Mrs. L. Hoch is gratefully acknowledged.

## REFERENCES

1. Kunkel, H. G. 1965. Myeloma proteins and antibodies. *Harvey Lect.* **59**:219.
2. Grey, H. M., M. Mannik, and H. G. Kunkel. 1965. Individual antigenic specificity of myeloma proteins. *J. Exp. Med.* **121**:561.
3. Kunkel, H. G. 1970. Individual antigenic specificity, cross specificity and diversity of human antibodies. *Fed. Proc.* **29**:55.
4. Potter, M., R. Liebermann, and S. Dray. 1966. Isoantibodies specific for myeloma  $\gamma$ G and  $\gamma$ H immunoglobulins of BALB/c mice. *J. Mol. Biol.* **16**:334.
5. Potter, M., and R. Lieberman. 1967. Genetic studies of immunoglobulins in mice. *Cold Spring Harbor Symp. Quant. Biol.* **32**:187.
6. Kunkel, H. G., M. Mannik, and R. C. Williams. 1963. Individual antigenic specificity of isolated antibodies. *Science (Wash. D. C.)*. **140**:1218.
7. Oudin, J., and M. Michel. 1963. Une nouvelle forme d'allotypie des globulines  $\gamma$  du s rum de Lapin apparemment li e   la fonction et   la sp cificit  anticorps. *C. R. Hebd. Seances Acad. Sci. Ser. D Sci. Nat. (Paris)*. **257**:805.
8. Kelus, A. S., and P. G. H. Gell. 1968. Immunological analysis of rabbit antibody systems. *J. Exp. Med.* **127**:215.
9. Braun, D. G., and R. M. Krause. 1968. The individual antigenic specificity of antibodies to streptococcal carbohydrates. *J. Exp. Med.* **128**:969.
10. Hopper, J. E., and A. Nisonoff. 1971. Individual antigenic specificity of immunoglobulins. *Adv. Immunol.* **13**:58.
11. Eichmann, K., and T. J. Kindt. 1971. The inheritance of individual antigenic specificities of rabbit antibodies to streptococcal carbohydrates. *J. Exp. Med.* **134**:532.
12. Williams, R. C., Jr., H. G. Kunkel, and J. D. Capra. 1968. Antigenic specificities related to the cold agglutinin activity of gamma M globulins. *Science (Wash. D. C.)*. **161**:379.
13. Oudin, J., and G. Bordenave. 1971. Observations on idiotype of rabbit antibodies against Salmonella aobrtus-equi. *Nat. New Biol.* **231**:86.
14. Kunkel, H. G., V. Agnello, F. G. Joslin, R. J. Winchester, and J. D. Capra. 1973. Cross-idiotypic specificity among monoclonal IgM proteins with anti- $\gamma$ -globulin activity. *J. Exp. Med.* **137**:331.
15. Kuettner, M. G., A. Wang, and A. Nisonoff. 1972. Quantitative investigations of idiotypic antibodies. VI. Idiotypic specificity as a potential genetic marker for the variable regions of mouse immunoglobulin peptide chains. *J. Exp. Med.* **135**:579.
16. Eichmann, K. 1972. Idiotypic identity of antibodies to streptococcal carbohydrate in inbred mice. *Eur. J. Immunol.* **2**:301.
17. Briles, D. E., and R. M. Krause. 1972. Mouse antibodies to group A streptococcal carbohydrate: Use of idiotype to detect inbred strain specificity and to monitor spleen cell transfer in syngeneic mice. *J. Immunol.* **109**:1311.



18. Sher, A., and M. Cohn. 1972. Inheritance of an idiotypic response associated with the immune response to inbred mice to phosphorylcholine. *Eur. J. Immunol.* **2**:319.
19. Eichmann, K. 1973. Idiotypic expression and the inheritance of mouse antibody clones. *J. Exp. Med.* **137**:603.
20. Potter, M., and R. Lieberman. 1970. Common individual antigenic determinants in five of eight BALB/c IgA myeloma proteins that bind phosphorylcholine. *J. Exp. Med.* **132**:737.
21. Blomberg, B., W. R. Geckeler, and M. Weigert. 1972. Genetics of the antibody response to dextran in mice. *Science (Wash. D. C.)*. **177**:178.
22. Carson, D., and M. Weigert. 1973. Immunochemical analysis of the cross-reacting idiotypes of mouse myeloma proteins with anti-dextran activity and normal anti-dextran antibody. *Proc. Natl. Acad. Sci. U. S. A.* **70**:235.
23. Oudin, J., and P. A. Cazenave. 1971. Similar idiotypic specificities in immunoglobulin factors with different antibody functions or even without detectable antibody function. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2616.
24. Braun, D. G., E. Kjems, and M. Cramer. 1973. A rabbit family of restricted high responders to the streptococcal Group A-variant polysaccharide. Selective breeding narrows the isoelectric focusing spectra of dominant clones. *J. Exp. Med.* **138**:645.
25. Bokisch, V. A., D. Bernstein, and R. M. Krause. 1972. Occurrence of 19S and 7S anti-IgGs during hyperimmunization of rabbits with streptococci. *J. Exp. Med.* **136**:799.
26. Krause, R. M., and M. McCarty. 1962. Studies on the chemical structure of the streptococcal cell wall. II. Composition of the Group C cell walls and the chemical basis for serological specificity of the carbohydrate moiety. *J. Exp. Med.* **115**:49.
27. 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.
28. Awdeh, Z. L., A. R. Williamson, and B. A. Askonas. 1969. Isoelectric focusing in polyacrylamide gel and its application to immunoglobulins. *Nature (Lond.)*. **219**:66.
29. Braun, D. G., and J.-C. Jaton. 1973. The aminoterminal sequence of antibody light chain: evidence for possible inheritance of structural genes. *Immunochemistry*. **10**:387.
30. Gold, E. R., and H. H. Fudenberg. 1967. Chromic chloride: a coupling reagent for passive hemagglutination reactions. *J. Immunol.* **99**:859.
31. Nisonoff, A., F. C. Wissler, L. N. Lipman, and D. L. Woernley. 1960. Separation of univalent fragments from bivalent rabbit antibody molecules by reduction of disulphide bonds. *Arch. Biochem. Biophys.* **89**:230.
32. Porter, R. R. 1959. The hydrolysis of rabbit  $\gamma$  globulin and antibodies with crystalline papain. *Biochem. J.* **73**:119.
33. Hunter, R. 1970. Standardization of the chloramine-T method of protein iodination. *Proc. Soc. Exp. Biol. Med.* **133**:989.
34. Braun, D. G. 1973. Monoclonal antibodies to streptococcal polysaccharides in rabbit and mouse. *Protides Biol. Fluids Proc. Colloq. Bruges*. **20**:163.

35. Jaton, J.-C., D. G. Braun, A. D. Strosberg, E. Haber, and J. E. Morris. 1973. Homogeneous antibodies: amino acid sequence of rabbit H chains of allotype a1, a2 and a3 in the region 80-94. *J. Immunol.* In press.
36. Kelus, A. S., and P. G. H. Gell. 1967. Immunoglobulin allotypes of experimental animals. *Prog. Allergy.* **11**:141.
37. Cramer, M., E. B. Jacobson, and D. G. Braun. 1973. Mouse antibodies with electrophoretic restriction. *Protides Biol. Fluids Proc. Colloq. Bruges.* **20**:193.
38. Hopper, J. E., A. B. MacDonald, and A. Nisonoff. 1970. Quantitative investigations of idiotypic antibodies. II. Nonprecipitating antibodies. *J. Exp. Med.* **131**:41.
39. Christian, Ch. L. 1963. Rheumatoid factor properties of hyperimmune rabbit sera. *J. Exp. Med.* **118**:827.
40. 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.
41. Potter, M., and H. G. Kunkel. 1971. Idiotypic antibody. *In* Progress in Immunology. B. Amos, editor. Academic Press, Inc., New York. **1**:1361.
42. Kindt, T. J., R. K. Seide, V. A. Bokisch, and R. M. Krause. 1973. Detection of idiotypic cross-reactions among streptococcal antisera from related rabbits. *J. Exp. Med.* **138**:522.
43. Aho, K., and O. Wagner. 1961. Production of "anti-antibodies" in rabbits. *Ann. Med. Exp. Biol. Fenn.* **39**:79.
44. Milgrom, F., and E. Witebsky. 1960. Studies on the rheumatoid and related serum factors. *J. Am. Med. Assoc.* **174**:138.
45. Williams, R. C., and H. G. Kunkel. 1963. Antibodies to rabbit gamma-globulin after immunizing with various preparations of autologous gamma-globulin. *Proc. Soc. Exp. Biol. Med.* **112**:554.
46. Eichmann, K., D. G. Braun, and R. M. Krause. 1971. Influence of genetic factors on the magnitude and heterogeneity of the immune response in the rabbit. *J. Exp. Med.* **134**:48.
47. Boyer, S. H. 1972. Extraordinary incidence of electrophoretically silent genetic polymorphism. *Nature (Lond.)*. **239**:453.
48. Kindt, T. J., D. G. Klapper, and M. D. Waterfield. 1973. An idiotypic cross-reaction between allotype a3 and allotype a negative rabbit antibodies to streptococcal carbohydrate. *J. Exp. Med.* **137**:636.
49. Wu, T. T., and E. A. Kabat. 1970. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J. Exp. Med.* **132**:211.
50. Westergaard, M., and D. von Wettstein. 1972. The synaptinomal complex. *Annu. Rev. Genet.* **6**:71.