IMMUNOLOGICAL ANALYSIS OF RABBIT ANTI-ANTIBODY SYSTEMS

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The phenomenon of "anti-antibodies" of individual (idiotypic) specificity was first described by Kunkel et al. (1) as a result of the experiments in which heteroprecipitins against human antibodies were employed. A similar finding of idiotypic determinants within the same (rabbit) species was reported by Oudin and Michel (2) and independently by Gell and Kelus (3). The terms "idiotypic" specificity as well as "isotypic" and "allotypic" specificities were proposed by Oudin (4) as simple and logical: "Three kinds of antigenic specificities with different genetic meanings may be distinguished . . . in immunoglobulins . . . The *isotypic* specificities are those which are uniform in all individuals of one animal species" (often referred to as species-specific determinants). "The *allotypic* specificities are those which are different in different groups of individuals within the same species" (these are usually spoken of as stimulating isoantibodies, though we should prefer the term antiallotypic antibodies). "The term *idiotypic* specificities was chosen to designate antigenic specificities of immunoglobulins which are peculiar in two respects. Each of them is peculiar, first to antibodies against one given antigen, and secondly, to one individual or perhaps to one group of individuals, within which the idiotypic specificities of the antibodies against one antigen is not the same as it is in other individuals or groups." All these specificities may be demonstrable by heteroimmunization, although the isotypic specificities are dominant: the allotypic specificities characteristic of many different kinds of molecule, or e.g. immunoglobuln class, will be demonstrable best by intraspecies (mouse into mouse, rabbit into rabbit etc.) immunization (see review by Kelus and Gell, reference 5). If pairs of animals which are genetically alike with respect to their allotypic constitution are cross-immunized with antibodies, the idiotypic specificities alone will be active in producing anti-antibodies(antiidiotypic antibodies). It should be noted however, as will be seen from the results described below, that unknown allotypic systems present in immunoglobulin classes can cause some complications.

The work to be described is mainly devoted to the investigation of anti-idiotypic antibodies and idiotypic determinants; these in our experience are strictly individual-specific and therefore the saving clause used in the quotation above from Oudin "or perhaps to one group of individuals"—may not be necessary.

Fig. 1. illustrates diagrammatically the properties of antiidiotypic antibodies in the system we have used. For clarity, we retain in the description of our

results the convention that the immunogenic donor antibodies (containing the idiotypic determinants) are called "D susbstance" or "D antibodies" and are recorded in Roman numerals; the antiidiotypic antibodies (anti-antibodies) in the recipients are called "R substance" or "R antibodies" and recorded in Arabic numerals. In some cases of cross-immunizations this rule of notation could not be followed.

Materials and Methods

Rabbits were especially bred for this investigation in our colony and were closely related. They originated from several European breeds.

The strain of *Proteus vulgaris X 19*, nonmotile, was cultured on nutrient agar for 24 hr, collected into formol saline (0.4% formaldehyde made up in 0.9% NaCl and buffered at pH 7.2). The suspension was heated in a water bath at 60°C for an hour, tested for sterility, made up to 10^{10} cells per ml, and stored at +4°C for several months.

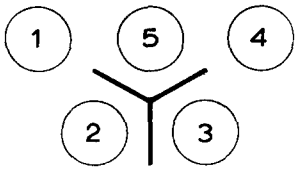

FIG. 1. Diagram of immunodiffusion: well 1, preimmunization sample of antiidiotypic antiserum (R); well 2, idiotypic antiserum (D); well 3, antiidiotypic antiserum (R); well 4, preimmunization sample of idiotypic antiserum (D); and well 5, proteus antigen.

Note: antisera 2 and 3 both have anti-proteus antibodies.

Before use, the organisms were thoroughly washed in PBS (phosphate-buffered saline: 0.9% NaCl, $\frac{1}{15}$ M phosphate buffer at pH 7.2). Immunization was carried out by intravenous injections biweekly, each injection containing 10^9 increasing to 10^{10} cells per dose: one course consisted of five injections (2×10^{10} cells in all).

The animals were bled 1 wk after the last injection and the sera stored at −20°C.

For anti-proteus sera one such course was sufficient; the sera were tested by immunodiffusion against an aqueous extract of freeze-dried proteus cells (100 mg extracted with 1 ml water or PBS).

To raise antiallotypic or antiidiotypic antisera unwashed proteus cells were coated with an excess of anti-proteus antibodies, usually 0.2 ml antiserum for 10^{10} cells. The suspension was incubated at 37°C for an hour or longer, then washed three times by centrifugation in PBS without sterile precautions, resuspended in sterile saline (to avoid any pyrogenic reaction), and injected using the same course as described above.

Two or more courses were usually necessary to produce such antibodies, with an interval of 3–4 wk between the courses.

Immunodiffusion was performed in 1% agar or agarose prepared in PBS and developed at room temperature for 18–48 hr.

Immunoelectrophoresis was performed in 1% agar prepared in barbitone buffer (0.05 M and pH 8.6), usually at 15 v/cm for 1–2 hr at constant voltage.

Papain and pepsin digests were prepared according to the methods of Porter (6) and of Nisonoff et al. (7) respectively.

Column fractionation was performed on Sephadex G-200 using 2 × 80 cm columns, in 0.5 M NaCl; 2 ml fractions were collected. The peak samples were concentrated 10–30 times by negative pressure dialysis.

Ultracentrifuge sucrose gradients were kindly carried out by Dr. D. Normansell of this Department: 0.25 ml sample was layered onto a sucrose density gradient 10–30%. This was sedimented at 39,000 rpm for 18 hr at +4°C in a Spinco preparative ultracentrifuge. The distribution of protein in the gradient was tested by injecting 60% sucrose through the base of the tube and analyzed in a Gilford automatic effluent analyzer. The effluent was collected in 11 drop fractions (34 fractions per 5-ml tube). They were tested in immunodiffusion agar plates.

Absorption of idiotypic (D) and antiidiotypic (R) antisera (see Table III) were carried out using 1 ml antiserum and 2×10^{10} proteus cells, twice or three times; the mixtures were incubated for 30 min at 37°C, centrifuged, and the supernatants tested by immunodiffusion.

Preparations of idiotypic antisera (D) for coating proteus cells to use in further absorptions (see Table IV) were made by mixing 2 ml of the appropriate antiserum (D) with 10^{12} proteus cells, incubated for 1 hr at 37°C, and washed very thoroughly. The coated proteus cells were then resuspended in 1 ml PBS and mixed with 1 ml of antiidiotypic antiserum (R) or with its dilutions, incubated for 1 hr at 37°C, centrifuged, and the supernatant used for testing by immunodiffusion.

RESULTS

All the rabbits were immunized by the standard technique, by means of killed *Proteus vulgaris X 19* coated with antibody. Table I summarizes the results on a large number of such immunizations. This table includes the results of an examination of 20 routine antiallotypic antisera, many of which are seen to contain antiidiotypic antibodies (R) specific to the original idiotypic immunoglobulin (anti-proteus antibody) of the donor (D). In addition, a number of immunizations resulted in the production of antiallotypic antibodies to rabbit macroglobulin; details of these systems will be published separately.[1]

In four cases only, autoimmunization was attempted and no antiidiotypic antibodies (R) were produced, after three courses.

In two antisera raised against As 3 (third to fifth course), in which the antiallotypic antibodies were weak—As 3 tends always to be poorly immunogenic —the antiidiotypic antibodies were appreciably stronger.

It should be emphasized that these data do not exclude unsuccessful immunizations in this system: in no case did we fail to produce some antiidiotypic antibody (R). One individual did not respond after three courses of immunization; this animal was rested for a year and was found to respond after one further course. There is thus no difficulty in raising anti-antibodies in rabbits and they are likely to be complicating factors in many isoimmunizations with antibody globulins.

In Table II results are set out on 10 of the strongest antiidiotypic antisera (R) against 60 individual anti-proteus antisera, including the original idiotypic antiserum (D). It can be seen that four of these contain additional weak

[1] Kelus, A. S. 1966. Unpublished data.

TABLE I

Summary of Immunization Results

Immunization				Antibodies produced			
D		R		Anti-*Proteus vulgaris*	Anti-idiotypic	Anti-allotypic	
Rabbit No.	IgG allotype	Rabbit No.	IgG allotype			Specific to IgG	Specific to IgM
I	As1/4	I	As1/4	+	−	−	−
II	As3/4, 5	II	As3/4, 5	+	−	−	−
III	As1, 3/4	III	As1, 3/4	+	−	−	−
		1	As1, 3/4, 5	+	+	−	−
		2	As1, 3/4	+	+	−	−
IV	As1, 3/5	IV	As1, 3/5	+	−	−	−
		3	As1, 3/5	+	+	−	+
		4	As1, 3/5	+	+	−	+
		5	As 3/5	+	+	+	−
V	As1, 3/5	6	As1, 3/5	+	+	−	−
6	As1, 3/5	V	Aa1, 3/5	+	+	−	+
VI	As1/4	7	As1/4	+	+	−	−
		8	As1/4	+	+	−	−
		9	As3/4	+	+	+	−
8	As1/4	10	As3/4, 5	+	+	+	−
VII	As1, 3/4, 5	11	As1, 3/4, 5	+	+	−	+
		12	As1, 3/4, 5	+	+	−	+
VIII	As1, 3/4, 5	12	As1, 3/4, 5	+	+	−	+
IX	As1, 2/4	13	As1, 2/4	+	+	−	−
		14	As1, 2/4	+	+	−	−
X	As1, 2/4	15	As1, 2/4	+	+	−	−
		16	As1/4	+	+	+	−
		17	As3/4	+	+	+	−
		18	As1/4	+	−	+	+
		19	As3/4	+	+	+	+
		20	As1/4	+	+	+	+
XI	As3/4	21	As3/4	+	+	−	−
		22	As3/4	+	+	−	+
		23	As3/4, 5	+	+	−	+
		24	As2, 3/5	+	−	+	+

TABLE I—*Concluded*

Immunization				Antibodies produced			
D		R		Anti-*Proteus vulgaris*	Anti-idiotypic	Anti-allotypic	
Rabbit No.	IgG allotype	Rabbit No.	IgG allotype			Specific to IgG	Specific to IgM
25	As3/5	XI	As3/4	+	−	+	−
XII	As3/4	26	As1, 3/4	+	+	−	−
		27	As2, 3/4	+	+	−	+
27	As2, 3/4	28	As3/4	+	−	+	−
XIII	As1, 3/4	29	As1, 3/4	+	+	−	+
XVI	As1, 3/4	XIII	As1, 3/4	+	+	−	+
		1	As1, 3/4, 5	+	+	−	−
		2	As1, 3/4, 5	+	+	−	−
		30	As1, 3/4	+	−	−	+
XV	As1/4	31	As1/4	+	+	−	+
XIV	As1, 3/4	32	As1, 3/4	+	+	−	+
XVII	As1/4	33	As1/4	+	+	−	−
		34	As1/4	+	+	−	−
		35	As1/4	+	+	−	−
XVIII	As3/4	25	As3/5	+	−	+	−
		36	As1/4, 5	+	−	+	−
		37	As1/4	+	+	+	−
		38	As1/4	+	+	+	−
		39	As1/4	+	−	+	−
XIX	As1/4, 5	40	As1/4	+	+	+	−
		41	As1/4, 6	+	+	+	−
XX	As1/4, 6	42	As1/4	+	−	+	−
XXI	As3/4, 6	43	As3/4	+	−	+	−

Every number both Roman and Arabic indicates an individual rabbit. In general, donor (D) animals are recorded in Roman numbers (I, II, etc) and recipient (R) animals in Arabic numbers (1, 2, etc); though in some cases where cross-immunizations were done this rule of notation is not adhered to (+, indicates positive immunodiffusion reaction). The table includes 20 R animals (5, 9, 10, 16–20, 24, XI, 28, 25, 36–43) which were used for routine antiallotypic immunization: of these all produced antiallotypic, 11 produced in addition antiidiotypic, and four antimacroglobulin allotypic antibodies.

Immunizations between different animals of identical IgG allotype were done in 33 cases using in all 15 donor sera: 32 recipients produced antiidiotypic antibodies, 13 produced in addition antimacroglobulin allotypic antibodies. In one case (30) an antimacroglobulin allotypic antibody was produced in the absence of antiidiotypic antibody. No anti-IgA specific allotypic antibodies were observed, nor was any new IgG allotypic specificity identified.

In 3 cases (D VII, VIII → R 12: D III, XIV → R 1 and R 2) recipients were immunized with two donors. Four animals (1–4) were used for "autoimmunization": no antiidiotypic or antiallotypic antibodies were observed. All the R animals produced some anti-proteus antibodies.

antibodies directed against non-IgG allotypic determinants. All antisera, which we have described in Table I as positive for antiidiotypic antibodies (R), were tested against 12 or more anti-proteus antisera and showed a strict individual specificity for the homologous idiotypic substance (D).

The proteus antigens, against which antibodies in the donor antisera (D) are directed, appear to be numerous (Figs. 2 and 3). It should be noted that they all migrate towards the anode at pH 8.6.

TABLE II

Immunodiffusion Reactions of the 10 Strongest Antiidiotypic Antisera against 60 Individual Anti-Proteus Sera (Including the Immunogenic D Serum)

D and other anti-proteus sera	R									
	1	2	3	8	11	14	15	22	27	29
III	+	+	−	−	−	−	−	−	−	+
IV	−	−	+	−	−	−	−	−	−	−
VI	−	−	−	+	−	−	−	−	−	−
VII	−	−	−	−	+	−	−	−	−	−
IX	−	−	−	−	−	+	−	−	−	−
X	−	−	−	−	−	−	+	−	−	−
XI	−	−	−	−	−	−	−	+	−	−
XII	−	−	+	−	−	−	−	−	+	−
XIII	−	−	−	−	−	−	−	−	−	+
XIV	+	+	−	−	−	−	−	−	−	−
Fifty individual anti-proteus sera*	0	0	3	0	8	0	0	2	0	1

Note: R 1 and R 2 were immunized both with D III and with D XIV (cf. Table I).

* The figures in the last line of the table indicate numbers of positive reactions: these and the two anomalous reactions in the body of the table (D XII/R 3 and D III/R 29) indicate reactions of "nonidentity" with the antiidiotypic line and are due to antimacroglobulin allotypic antibodies.

In a number of cases (see Table I) a single anti-proteus antiserum (D) was injected into several recipients. The antiidiotypic antibodies (R1, R2, etc.) in these experiments had the same specificity; i.e., if two animals (R1, R2) were injected with an anti-proteus antibody (D), a reaction of identity resulted in gel diffusion (Fig. 4). On the other hand, since there are numerous anti-proteus antibodies in idiotypic antisera (D) against proteus components, one might expect in some cases that multiple antiidiotypic antibodies (R) could arise: this often happens and up to four separate precipitation lines have been observed. This phenomenon is illustrated in Figs. 5 and 6.

Similarly, if a mixture of anti-proteus antibodies from two different animals (D1 and D2) is injected into a single recipient (R), the antiidiotypic antibodies produced will react nonidentically with these two anti-proteus antisera (Fig. 7). Naturally, an antibody against a totally different antigen such as human

IgG, raised in a donor rabbit (D) after the disappearance of anti-proteus antibodies, shows no cross-reactivity with the antiidiotypic antibody (R) as demonstrated in Fig. 8.

Fig. 9 shows the reaction between selected idiotypic antigens and antiidiotypic antibodies, in the one case when the idiotypic substance (D) and in the other when the antiidiotypic antibody (R) is electrophoresed. This illustrates

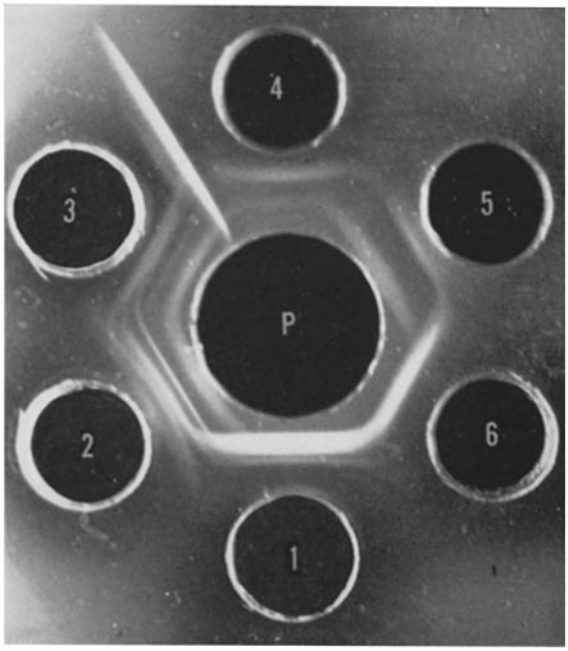

FIG. 2. Immunodiffusion reactions between individual anti-proteus antisera (1–6) and proteus extract (P).

Note: Antiserum 3 has in addition antiallotypic antibody against IgG determinants of antiserum 4.

both the high electrophoretic homogeneity of the idiotypic substances (D), which are anti-proteus antibodies, and a multiplicity of idiotypic-antiidiotypic reactions.

It is clearly desirable to demonstrate that the idiotypic substance (D) is actually an anti-proteus antibody, and not an epiphenomenon of immunization. Reactions of identity between the anti-proteus and proteus extract lines, and the idiotypic and antiidiotypic lines in an immunodiffusion plate (Fig. 10) go some way to demonstrate this. A more rigid test might be to show that absorption of the anti-proteus antibody (D) with excess of proteus antigens removes all reactivity with antiidiotypic antibodies (R). This is however complicated by the fact that the antiidiotypic antisera (R) themselves contain strong

antibodies to proteus antigens as well as to the idiotypic donor antisera (D), since in the nature of the case they have to be raised with the identical immunogen (proteus cells), coated with the anti-proteus antibody (D): and they are strong because the course of immunization is necessarily a long one. To absorb the anti-proteus antibodies from the antiidiotypic antisera (R) in addition, is not as simple as it sounds.

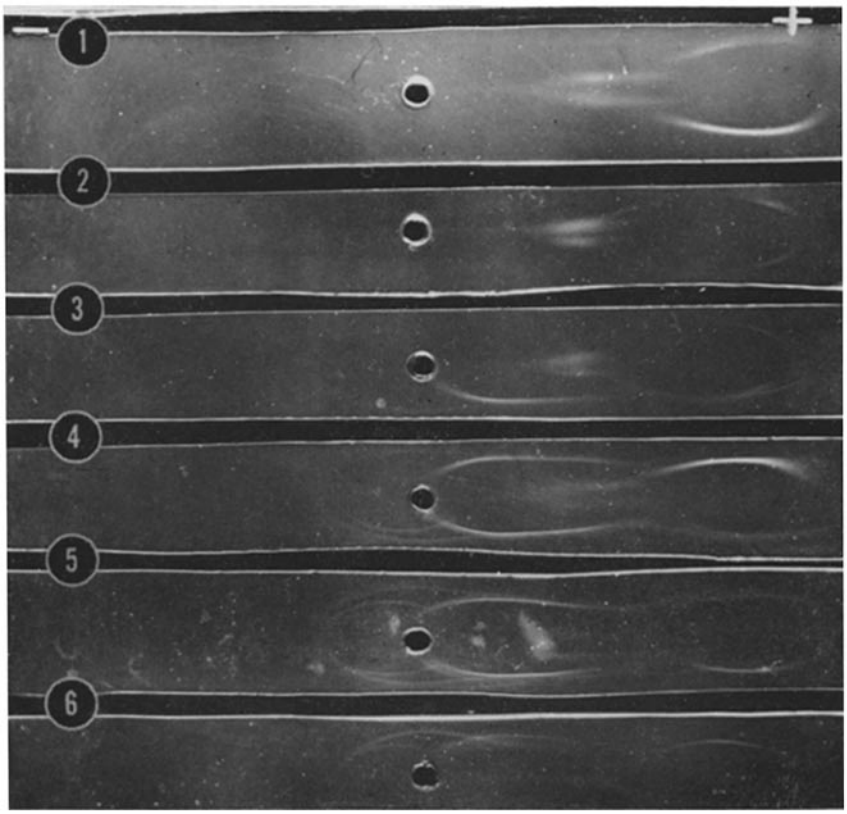

FIG. 3. Immunoelectrophoretic patterns of the reactions between individual anti-proteus antisera (1–6) and proteus extract.

Absorption of the idiotypic antisera (D), derived from a short course of immunization with *Proteus vulgaris X 19*, using washed proteus cells, will remove their reactivity to the antiidiotypic antisera (R) if these antisera (R) are absorbed in the same way. Both these absorbed antisera (D and R) will however contain small amounts of proteus antigens derived from the bacteria used for absorption; the antiidiotypic antisera (R) contain in addition some

unabsorbed antibodies; e. g., to nonsurface antigens of proteus (since these are long-course immunization sera, although with coated bacteria). Thus an absorbed antiidiotypic antiserum (R) will react with proteus extract, because of this antibody to the internal antigens of the bacteria: and the unabsorbed antiidiotypic serum (R) will react with its corresponding absorbed R serum, because of free proteus antigens which the latter contains. Since however these free antigens are the same both in the absorbed antiidiotypic antiserum (R) and in the absorbed idiotypic antiserum (D), they do not mediate a reaction between them. The situation is highly complex because of the multiplicity of antigens and antibodies involved. As far as the particular antisera illustrated in Table

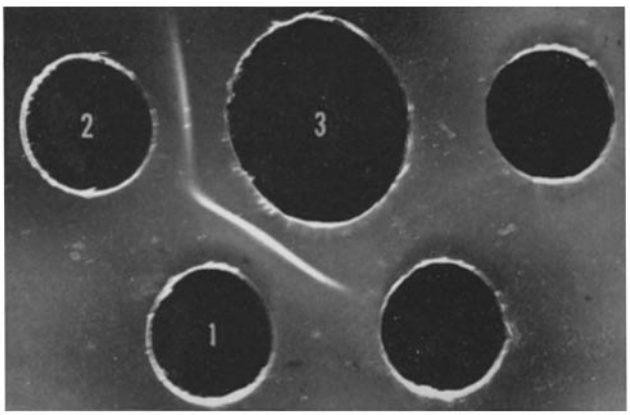

FIG. 4. Immunodiffusion reaction of identity between two individual antiidiotypic antisera (R) in wells 1 and 2, and the corresponding idiotypic antiserum (D) in well 3.

Note: The apparent spur in the precipitation line between 1 and 3 is continuous with a second faint line between 2 and 3, representing a second idiotypic system.

III, absorption of the idiotypic antisera (D) was successful. In the case of other (D) antisera, or of the (D) antisera in Table III with less complete absorption, reactions remained between antiidiotypic and idiotypic antisera (R and D) owing to incomplete absorption of the idiotypic antibodies (D).

Some clarification may be gained by examining the reactions of identity between the various precipitation lines of immunodiffusion plate (Fig. 11). However, a much more satisfactory system will be devised when a single purified antigen can be used for this analysis.

Further evidence as to the antibody nature of the idiotypic substance (D) has been obtained from physicochemical methods. Two idiotypic antisera (D) were filtered on Sephadex G-200. One antiserum showed activity in the second peak where IgG is found, as identified by a specific sheep anti-rabbit IgG antiserum. The other antiserum had multiple idiotypic (D) reacting substances: both the first and the second peaks reacted with the corresponding antiidiotypic

antiserum (R). The first peak reaction, identified by a specific sheep anti-rabbit IgM antiserum, was not to a macroglobulin allotypic determinant and suggested that an antiidiotypic antibody was directed against a macroglobulin antibody (D) in this anti-proteus antiserum. The second peak reaction was of IgG nature, again identified by the sheep anti-rabbit IgG antiserum. These two idiotypic antisera (D) were also ultracentrifuged in a sucrose gradient: activity was found

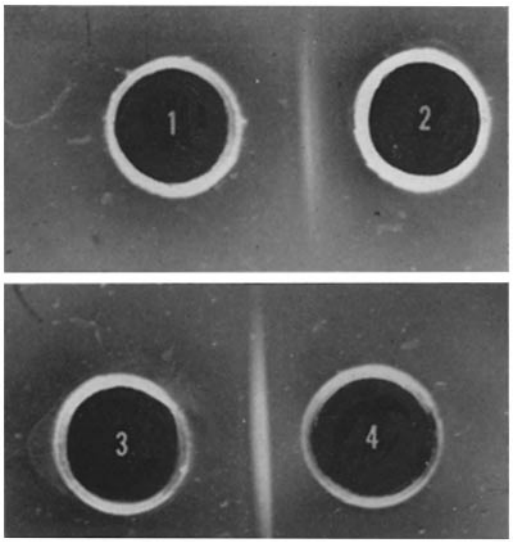

FIG. 5. Immunodiffusion reactions between idiotypic antisera (D) in wells 1 and 3, and antiidiotypic antisera (R) in wells 2 and 4.

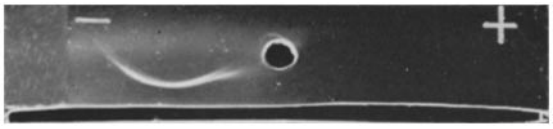

FIG. 6. Immunoelectrophoretic pattern of the reaction between idiotypic antiserum (D) in the well and its corresponding antiidiotypic antiserum (R) in the trough.

in the 7S fraction only; failure to detect the 19S substance (D) was probably due to quantitative reasons.

Papain and pepsin digestions of the IgG fractions from these two idiotypic antisera (D) were done. The reactivity with the corresponding antiidiotypic antisera (R) was found in both cases to survive digestion, and was present on the Fab fragment after papain treatment (Fig. 12).

These results would suggest that the determinant recognized by the anti-idiotypic antiserum (R) is on an antibody molecule, and is in the region of the

antibody-combining site. Is it the antibody-combining site itself? Clearly, if this were so, one would expect that reactivity of the idiotypic substance (D) should be eliminated by blocking the site with proteus antigens. Table IV illustrates an experiment which shows that this is not so. It can be seen that anti-idiotypic antibodies (R) can be removed readily by idiotypic substances (D)

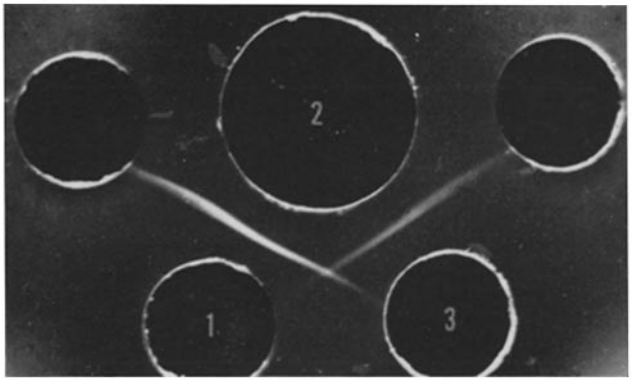

FIG. 7. Immunodiffusion reaction between two individual idiotypic antisera (D) in wells 1 and 3, and their corresponding antiidiotypic antiserum (R) in well 2.

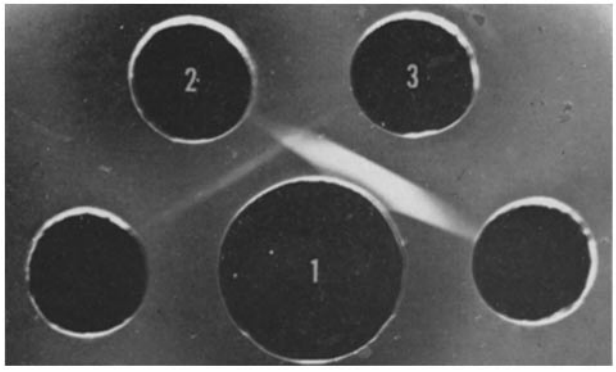

FIG. 8. Immunodiffusion reaction between an antiserum 1 and its corresponding antigens: idiotypic antiserum (D) in well 2 and human IgG in well 3.

complexed with proteus cells. Our previous results have indicated that the amount of bacterial cells used in this experiment (10^{12} organisms) is nearly 100 times the amount needed for complete removal of idiotypic substance (D) from these sera (see Table III); it is therefore most unlikely that any antibody-combining sites would remain free on the idiotypic (D) molecules.

The assumption that the idiotypic determinants recognized by the anti-idiotypic antibodies (anti-antibodies) are inherited in a simple Mendelian fash-

ion is difficult to make, since we have noted no cross-reactions whatever in these experiments, using closely interrelated rabbits from our laboratory stock. Nevertheless, we made what might be thought a rather naive attempt to check

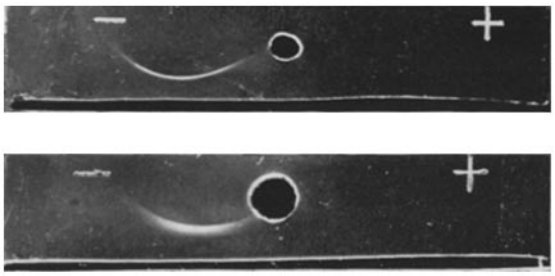

FIG. 9. Immunoelectrophoretic patterns of the reactions between: (*a*) an idiotypic antiserum (D) in the upper well and its antiidiotypic antiserum (R) in the upper trough; and (*b*) the same antiidiotypic antiserum (R) in the lower well and its idiotypic antiserum (D) in the lower trough.

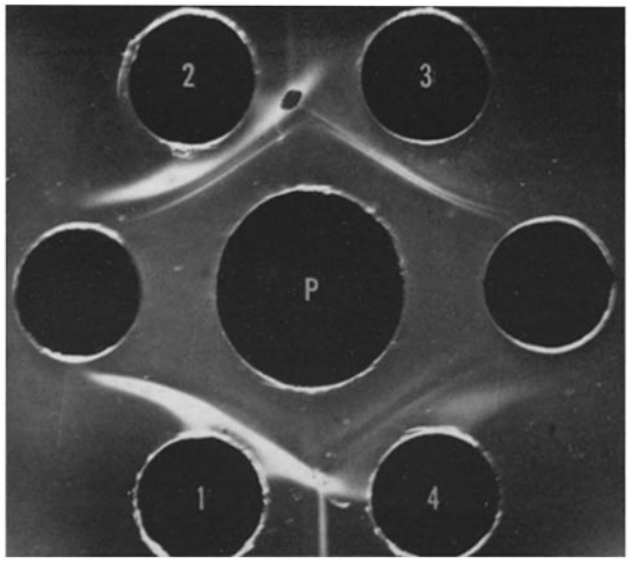

FIG. 10. Immunodiffusion reactions between proteus extract (P), two antiidiotypic antisera (R) in wells 1 and 2, and their corresponding idiotypic antisera (D) in wells 4 and 3; vertical precipitation lines are the R versus D reactions.

this. Two bucks were selected, which between them had fathered 30 offspring: all of these offspring and the bucks were simultaneously immunized with *Proteus vulgaris X 19*, and the parental anti-proteus antisera were successfully used to raise two antiidiotypic antisera (R). These (R) antisera showed no reaction

TABLE III

Absorption of R and D Antisera with Proteus Extract: Immunodiffusion Reactions

R serum absorbed	Reaction with			
	Proteus extract	D Serum absorbed	D serum unabsorbed	R serum unabsorbed
1	+	III –	+	+
		XIV –	+	
2	+	III –	+	+
		XIV –	+	
11	+	VII –	+	+
14	+	IX –	+	+
27	+	XII –	+	+

For explanation of these reactions see text.

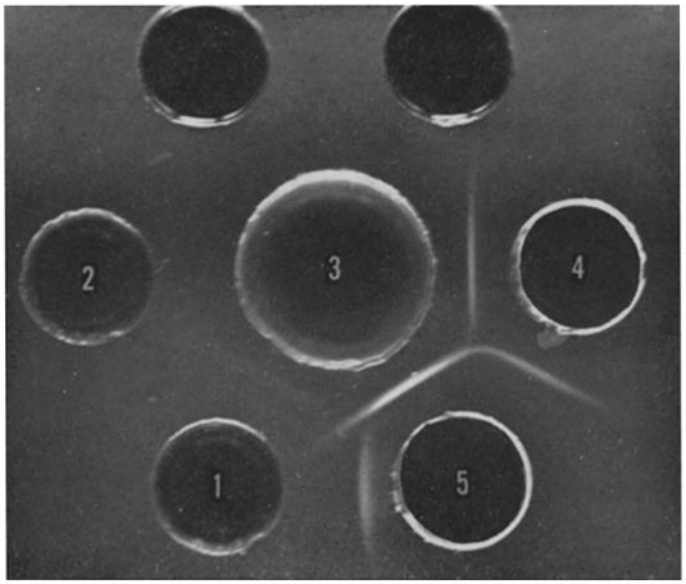

FIG. 11. Immunodiffusion reactions between: well 1, proteus extract; well 2, the idiotypic antiserum (D) absorbed with proteus cells; well 3, its corresponding antiidiotypic antiserum (R) absorbed with proteus cells; well 4, the same antiidiotypic antiserum (R) as in well 3 but unabsorbed; and well 5, the same idiotypic antiserum (D) as in well 2 but unabsorbed.

Note: The precipitation between wells 3 and 4 is due to the residual proteus antigens in the absorbed antiidiotypic antiserum (R).

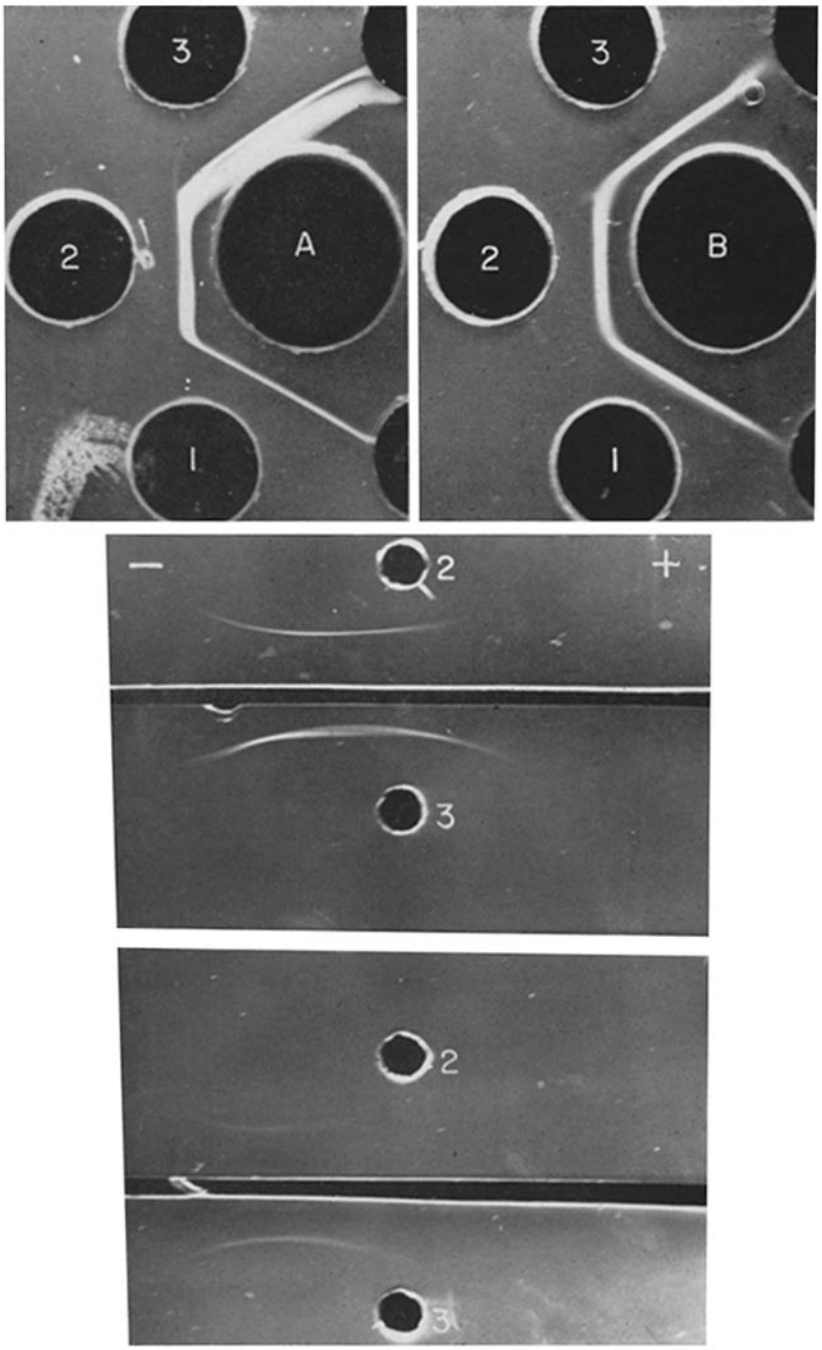

FIG. 12. Immunodiffusion and immunoelectrophoretic patterns of the reactions between antiidiotypic antiserum (R) and papain-digested idiotypic IgG (from antiserum D): A, goat anti-rabbit IgG; B, antiidiotypic antiserum (R); 1, idiotypic antiserum (D); 2, idiotypic IgG (from antiserum D); 3, idiotypic IgG (from antiserum D) digested by papain.

whatever with the anti-proteus antibodies produced by any of the progeny. This at least suggests that no simple pattern of inheritance is involved.

TABLE IV

Immunodiffusion Reactions of Two R Sera (2 and 27) Absorbed by Proteus Cells Coated with Homologous and Nonhomologous D Substances

R serum absorbed with proteus cells coated with D serum	D serum	Dilutions of R serum						D serum	Dilutions of R serum					
		1/2	1/5	1/10	1/20	1/40	1/80		1/2	1/5	1/10	1/20	1/40	1/80
Part *a*:														
Unabsorbed	No. III (homologous)	+	+	+	+	−	−	No. VII (nonhomologous)	−	−	−	−	−	−
Absorbed with No. III-coated cells (1:1)	" "	(±)	−	−	−	−	−	" "	−	−	−	−	−	−
Absorbed with No. III-coated cells (1:5)	" "	×	−	−	−	−	−	" "	−	−	−	−	−	−
Absorbed with No. III-coated cells (1:10)	" "	×	×	+	−	−	−	" "	−	−	−	−	−	−
Absorbed with No. XII-coated cells (1:5)	" "	×	×	+	+	−	−	" "	−	−	−	−	−	−
Absorbed with proteus cells only (1:5)	" "	×	×	+	+	−	−	" "	−	−	−	−	−	−
Part *b*:														
Unabsorbed	No. XII (homologous)	+	+	+	+	+	−	No. VII (nohomologous)	−	−	−	−	−	−
Absorbed with No. XII-coated cells (1:1)	" "	+	+	−	−	−	−	" "	−	−	−	−	−	−
Absorbed with No. XII-coated cells (1:5)	" "	×	−	−	−	−	−	" "	−	−	−	−	−	−
Absorbed with No. XII-coated cells (1:10)	" "	×	×	−	−	−	−	" "	−	−	−	−	−	−
Absorbed with No. III-coated cells (1:5)	" "	×	×	+	+	+	−	" "	−	−	−	−	−	−
Absorbed with proteus cells only (1:5)	" "	×	×	+	+	(±)	−	" "	−	−	−	−	−	−

In each case the same number of *Proteus vulgaris* cells and volume of undiluted or diluted R antiserum was used.
×, dilutions not tested.

DISCUSSION

Is the determinant against which so called anti-antibodies are directed really on an antibody molecule, or can it be an antigenic fragment or component of the original bacterial immunogen? This point has been argued in previous publications on this subject (2, 3) and we may repeat the argument here. The physicochemical properties of the idiotypic substance (D) such as observed in electrophoresis, gel filtration, and ultracentrifugation, show that it goes with IgG (or sometimes with IgM). In the two cases studied here, papain digestion results were consistent with the idiotypic substance (D) being on the Fab fragment of IgG. All proteus antigens, which we have been able to identify immuno-

logically, have been found in the anodic region (see Fig. 3) and would not be easily confused with IgG. Even if the mobility of such an antigen were altered by immunological complexing with the idiotypic antibody (D), that antibody would be "blocked": our results show that the idiotypic substance (D) is functional as precipitin.

Were there a particular antigen of *Proteus vulgaris X 19*—characteristically nonimmunogenic in the donor animals (D) and persistent in their antisera, but immunogenic in the recipient animals (R)—a single reaction between anti-idiotypic antiserum (R) and idiotypic substance (D) might possibly be explainable. But since there is never a reaction between the antiidiotypic antibody (R) and the "wrong" idiotypic serum (D), each of our 19 specific R-D interactions must be explained by a *different* persistent proteus antigen, unique to that particular D antiserum, from which all the other "persistent" proteus antigens happen to be excluded. This is clearly even less likely than the occurrence of anti-antibodies.

The same arguments apply to suggestions that the determinant (D) may be a complement component, C-reactive protein, or some other electrophoretically negative serum component, unless each rabbit has produced such a component which was individual-specific. From such arguments and from the evidence of reactions of identity between lines given by the idiotypic substance (D) as antibody reacting with proteus and as antigen reacting with the antiidiotypic antiserum (R), we think that it is established that the determinant (D) "recognized" by the antiserum (R) is indeed an antibody molecule.

We report here a total lack of cross-reaction, where different immunogens (D) are used, between some 60 actual or potential idiotypic substances (D) and 10 antiidiotypic antisera (R): within these quantitative limits, therefore, we feel that we may postulate that antibody produced by each animal has a unique individual specificity. Moreover, since there is a similar lack of cross-reaction when a recipient (R) animal "recognizes" several idiotypic substances (D determinants) on different antibodies in a given antiserum (D), and since other antibodies produced later by this animal (D) to other antigens are non-reactive with the antiidiotypic antiserum (R), we can postulate, though with less quantitative force, that every antibody in a given animal has a set of unique specific determinants different from all other antibodies in that animal.

In these circumstances there would seem to be one or more real chemical determinants characteristic of every antibody: and these determinants can be specifically recognized by some other animals—possibly after prolonged immunization by all animals—exposed to them, as shown by the reaction of identity between different antiidiotypic antisera (R) to a single idiotypic substance (D). It is true that not all antibodies against proteus antigens have been shown to provoke anti-antibodies though prolonged immunization increases the number of anti-antibodies produced. The reasons for this may be merely quan-

titative or, what amounts to the same thing in the long run, due to lack of homogeneity of some of the anti-proteus antibodies, especially those against multideterminant protein components (see below).

Is the idiotypic determinant (D) identical with the antibody-combining site? Two arguments may be used against this. Firstly, although many idiotypic antibodies (D) are directed against an identical proteus component, antiidiotypic antibodies (R) raised against them do not, as has been said, cross-react. Since the "shape" of the (D) anti-proteus site must be determined by the shape of the proteus antigen, one would expect some correspondence between the "shapes" of the antibody-combining sites on different antiidiotypic (R) molecules which would react with this structured combining site on the idiotypic (D) antibody. Secondly, blocking the site with excess of proteus antigen does not significantly affect the ability of the idiotypic substance (D) to react with the antiidiotypic antibody (R). Though such experiments are not completely conclusive, one would expect some steric interference with combination in these circumstances.

It is of course the case that in the immunogenic suspension (bacteria coated with antibody) the antibody-combining site is also blocked with antigen. If therefore the whole complex of the whole antibody (D) plus proteus antigen is the ultimate immunogen, presumably after binding in or on the macrophage, then the antibody-combining site itself might be unavailable for stimulating an immune response; or if some "processing" of the complex by the macrophage occurs, the site may be damaged thereby. It is also possible that the properties of the site may be due to a tertiary structure which is too labile to immunize as such. The evidence so far suggests that the idiotypic determinant (D) is in some way dependent upon the nature of the binding site and is, in some cases at least, on the Fab piece and therefore may be located on the variable portion of the L and/or H chain. We cannot however say that the idiotypic determinant (D) is never on the other parts of the IgG molecule; e.g., the Fc piece of the H chain.

Since the immunogen (idiotypic determinant D) for the antiidiotypic antibody (R) is itself an antigen-antibody complex, is the specificity recognized by the antiidiotypic antibody an "exposed" determinant resultant from molecular distortion in the reaction as in the case of the less specific anti–complex antibody described by Henney et al. (8)? As far as the antigenic properties of the idiotypic antibodies (D) are concerned, we can state that this is not so, since the unreacted and undistorted antibody (D) in the antiserum will precipitate with the antiidiotypic antibody (R). But it is possible that the *immunogenicity* may be partially dependent upon molecular distortion—we have no evidence that uncomplexed antibodies (D) will ever provoke antiidiotypic antibodies (R). This is a possibility quite independent of the nonspecific adjuvant action of the endotoxin contained in *Proteus vulgaris* X 19, which may or

may not be essential. Any definite conclusion on this point must await the results of further experimentation.

Is the idiotypic determinant (D) a chemical sequence arising in an animal de novo as a result of antigenic stimulation? Here we touch, of course, upon one of the fundamental problems of immunology. The only point which can be discussed profitably concerns the problem of autotolerance, upon which our quantitative data are scanty. In four rabbits only described here, autoimmunization was attempted under conditions which were successful for isoimmunization, and no antiidiotypic antibodies (R) were produced. So for what it is worth one could suggest that animals are tolerant to their own antibodies; this would not be unexpected, though the mechanism of this sort of autotolerance is difficult to explain. It should be emphasized that the idiotypic determinants (D) are quite strongly immunogenic: their effectiveness in an isoimmunization situation is quite comparable with, for example, the H chain allotypic markers. It would seem therefore that some form of autotolerance is necessary. A clonal elimination theory however would entail the elimination of a number of clones equivalent to the total number of possible antibodies, which seems rather radical though a "low-dose tolerance" theory might be satisfactory.

If autotolerance does exist, it is an example of tolerance of an extremely high specificity, in view of the fact that we were 100% successful in our attempts to raise antiidiotypic antibodies (R) by isoimmunization.

It remains to discuss the question of the homogeneity of the idiotypic antibodies (D). It is obvious that most so called specific antibodies even after purification, are highly complex mixtures, those against proteins being directed against perhaps 20 or more quite different antigenic determinants. It seems that quite by chance investigators who demonstrated antiidiotypic antibodies (anti-antibodies) have used systems which, though complex, contain some antigens which are "monodeterminant" and stimulate the production of antibodies which are highly homogeneous. Very often these seem to be directed against carbohydrate antigens, as it was with those demonstrated by heteroimmunization against human antibodies (1). A very striking case of a highly homogeneous antibody to streptococcal carbohydrates has been reported by Osterland et al. (9). It is not surprising that a homogeneous antibody will have a higher concentration of a particular idiotypic determinant (D) and because of that it will be more immunogenic than an apparently much stronger antibody which is really a mixture of many molecular species. Similarly, the phenomenon of "deletion" or "predominance" of allotypic determinants in antibodies (see reference 5) should be much more readily demonstrable in such homogeneous antibody populations. The evidence for electrophoretic homogeneity, which we have presented, in the idiotypic (D) antibodies suggests that deletion may occur here, and we have some preliminary data that this is so.

Further evidence for homogeneity of the idiotypic substance (D) is presented

by Feinstein and Kelus.[2] The IgG of the idiotypic antiserum (D) was isolated by salt precipitation and then partially reduced with mercaptoethanol and blocked with iodoacetamide. The reduced idiotypic substance (D) was then precipitated with antiidiotypic antibody (R), washed, dissolved in 8 M urea, and run on starch block at an alkaline pH. Under these circumstances the idiotypic substance (D), but not the antiidiotypic antibody (R), splits its constituent chains. The L chains derived from the idiotypic substance (D) appeared as a single band, and the H chains as two resolved bands, in both cases being much more homogeneous than would be expected from "normal" IgG.

Is there any evidence that idiotypic specificities (D) are present in germ lines and inherited in Mendelian fashion? The absence of any cross-reactions in the experiments described, using rabbits from our laboratory stock which were known to be closely interrelated, is against this. The more direct, though still not conclusive test, in which the anti-proteus antibodies (D) of parents and offspring were compared with respect to their idiotypic specificities, was negative. If the idiotypic specificities (D) were in any way heritable one could expect that the anti-proteus antibodies of at least some of the progeny would react with these anti-parental idiotypic antisera (R). None in fact showed any reaction, which suggests that no simple pattern of inheritance (if any) is involved.

Such systems, where molecular homogeneity is easily demonstrable and where the homogeneous population of molecules can be readily and specifically separated from the heterogeneous normal IgG, are clearly ideal for analysis in terms of precise protein structure and its correlation with gene action. These molecules have the advantage over, for instance, the myeloma proteins in that they are produceable at will in different genetic situations, and can throw light upon the highly complex problem of the relation of genetic factors to antibody production.

SUMMARY

A study has been made of the production of antiidiotypic antibodies (anti-antibodies) arising during the immunization of 39 rabbits with 19 individual samples of rabbit anti-*Proteus vulgaris* X 19 antibodies adsorbed onto bacilli. In addition to the regular demonstration of antiidiotypic antibodies attention is drawn to the frequent occurrence of antiallotypic antibodies against molecules of immunoglobulin classes other than IgG, especially macroglobulins, which may arise during such immunization. In four cases attempts to raise autoantiidiotypic antibodies were unsuccessful, as expected.

The idiotypic specificities (antigenic determinants) have been found mainly on IgG but also sometimes on IgM molecules. The individual specificity of the antiidiotypic antibodies appears to be absolute, as long as the antiallotypic

[2] Feinstein, A., and A. S. Kelus. 1966. Unpublished data.

antibodies are recognized and excluded; e.g., each idiotypic specificity is characteristic of only one single rabbit and of a single kind of antibody within that rabbit. These principles hold even when antibodies of rabbit families are examined: the parental idiotypic determinants could not be found in the offspring.

In two samples tested the idiotypic specificities were found on the Fab fragment of IgG molecule but not on its antibody-combining site.

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