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Antigenic Structure of Rabbit Gamma Globulin

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Summary. By iso-immunization, antisera to five rabbit γ globulin antigens were obtained. They are called A (former D^a), B, C, D and E. Individual sera of 670 rabbits belonging to six separate populations were tested by precipitation methods. The distribution of the iso-antigens and their combinations into serum groups were studied. Each particular γ globulin iso-antigen was found to be of hereditary character; they seem to form three genetic systems: A, C and BDE, statistically independent.

Various antisera from England, Poland and U.S.A. were compared.

INTRODUCTION

Iso-immunization has generally proved to be the best method of examining group antigens within a species. Owing to the 'faulty perspective' (Landsteiner, 1947) the method of hetero-immunization is without any doubt less refined and accurate than iso-immunization. Nevertheless, hetero-immunization was until recently the only method for the study of serological specificity of serum proteins. The attempts at experimental production of iso-precipitins were unsuccessful and the early work of Schütze (1902), who obtained iso-precipitins for rabbit serum proteins, was not confirmed for a long time.

Oudin (1956) used adjuvants and produced iso-precipitins by injecting rabbits with precipitates composed of rabbit antibodies and various antigens together with adjuvants. These iso-precipitins varied as to specificity; using six antisera it was possible to group forty-five rabbit sera into nine types. Oudin's results were confirmed and extended by Dray and Young (1958) who injected rabbits with whole rabbit sera mixed with adjuvants. They grouped ninety rabbit sera into thirteen types; the serum antigens were of α, β and γ globulin electrophoretic mobility. In their recent paper Dray and Young (1959) identified two rabbit γ globulin iso-antigens: RGG I and RGG II.

Working independently Dubiski, Dudziak, Skalba and Dubiska (1959) obtained iso-precipitins in rabbits immunized with bacteria which had been agglutinated with rabbit antibacterial sera. Using these iso-precipitins they divided the rabbit sera into two groups: D(a+) and D(a−). The D^a γ globulin antigen was shown to be of hereditary character. Furthermore, the D^a antigen was transmitted directly and non-genetically from mother to offspring, but was then eliminated, so that the adult rabbits possessed only genetically determined antigens. The authors grouped ninety-two sera of adult unrelated rabbits. Group D(a+) was found in 27 per cent.

During our first experiments it was observed that reactions of the sera with various antisera earlier designated as anti-D^a were not parallel. It seemed probable that the observed phenomena involved, besides the D^a — anti-D^a, some other γ globulin antigen-antibody systems. Further experiments revealed the existence of such systems and many new antisera of differing specificity were obtained. A detailed analysis of the antigens and antibodies concerned was performed.

MATERIALS AND METHODS

PRODUCTION OF ANTIBACTERIAL SERA. For the production of antibacterial sera rabbits of three populations were used: Rokitnica, Big R and Pathology. The rabbits were immunized with killed bacterial cells such as *Proteus vulgaris X* 19, *Salmonella typhi-murium* or *Streptococcus pyogenes*. Each rabbit was given five to ten intravenous or intraperitoneal injections of 1 ml. of a bacterial suspension containing approximately 10^8-10^9 micro-organisms. The animals were injected twice a week and bled 10 days after the last injection.

PRODUCTION OF ISO-PRECIPITINS. The antibacterial sera were used for the production of iso-precipitins. Each animal was immunized with only one antibacterial serum. One drop (0.03 ml.) of a bacterial suspension containing approximately 10^9 cells was mixed with 1 ml. of homologous serum (previously heated for ½ hour at 56°). The mixture was incubated for 60 minutes at 37° (in water bath), and the bacteria were then washed three times with saline. Since during the incubation and centrifugation procedure a strong agglutination occurred, the suspension was shaken vigorously before injection. The inoculum thus prepared was injected into the rabbits intravenously or intraperitoneally at intervals of 3 days or a week. After ten to fifteen injections the serum was checked for the presence of antibodies by precipitation in tubes and agar gel. Sometimes several courses of immunization were performed. Blood was taken from the animals 10 days after the last injection. All sera were stored at −20°.

ANALYSIS OF ISO-PRECIPITINS. The iso-precipitins were identified by testing against a panel of rabbit sera of known antigenicity in either tube or agar-gel precipitation. The antigen-antibody systems were then further analysed immunoelectrophoretically (Grabar and Williams, 1953).

The agar-gel precipitation tests were performed by the method of Ouchterlony (1949). A solution containing 1.5 per cent agar, and sodium azide or merthiolate in buffered saline was employed. The distance between the edges of neighbouring wells was 7.5 mm. or 10 mm. The wells were filled with undiluted antiserum, and with the sera to be tested either undiluted or diluted 1:3. The plates were then left in a wet chamber at room temperature and read after 4-7 days.

NOMENCLATURE. In the present investigations several γ globulin iso-antigens were found. We designated the antigens by capital letters in alphabetical order: A, B, C, D, and E. Antisera to these antigens were named anti-A, etc. The antigen D^a described previously by Dubiski, Dudziak, *et al.* (1959) was called A. Small letters: a, b, c, d and e were used to designate absence of the corresponding antigens. (The genetic systems are not yet well enough established to be specially named.)

RABBIT POPULATIONS. The γ globulin iso-antigens were studied in 670 individual rabbit sera belonging to six separate populations: Rokitnica, Big R, Veterinary, Pathology, RSH and RSJ. The population Rokitnica was composed partly of randomly selected

rabbits. Some of the animals in this population were related. The populations Big R, Veterinary and Pathology were cross-bred, while the others (RSH and RSJ) were random mating strains started in Cambridge by Dr. J. Hammond many years ago.

RESULTS

Analysis of antigen-antibody systems

Using the described methods of immunization, we obtained twenty-two precipitating antisera, some of which are listed in Table 1.

Table 1

Antisera used in the present work

Antiserum		*Obtained by injection of rabbit antibody*			*Antibody present in serum*
No.	*Antigens*	*No.*	*Antigens*	*Bacteria*	
176*	aBCDE	569	AbcDE	Salmonella typhi-murium	anti-A
761	abcdE	453	AbcDe	Proteus vulgaris *X* 19	,,
R-3292	abcDE	R-55/58	AbcDe	,,	,,
60	abcdE	175	ABcDe	Streptococcus pyogenes	anti-A+unidentified
573	abcDe	896	,,	Salmonella typhi-murium	anti-A+anti-B
R-3293	,,	R-3297	aBcDe	Proteus vulgaris *X* 19	anti-B
R-3294	,,	,,	,,	,,	,,
56	abcDE	880	aBcdE	,,	,,
186	AbcDE	174†	AbCDe	Streptococcus pyogenes	anti-C
198	abcDe	,,	,,	,,	anti-C+anti-A (trace)
R-3229	abcdE	R-55/58	AbcDe	Proteus vulgaris *X* 19	anti-A+anti-D
482	AbcDe	559	abcDE	,,	anti-E

* Serum 176 was used in the first paper of this series (Dubiski, Dudziak *et al.*, 1959) as anti-D^a.
† Serum 174 did not exhibit any precipitation with anti-A. The amount of the antigen A in this serum, estimated by the inhibition of haemagglutination, was approximately 200 times smaller than in other A sera.

The unknown antiserum was first tested against panel sera in agar gel and the reactions compared with those of known antisera. Any variation in the type of reaction (e.g. strength) was noted. Then the tested antiserum was submitted to neutralization experiments with various sera that contained known antigens. If the antiserum could not be

identified with any of the known reagents, the antiserum under study was assigned a 'new' specificity. When the serum contained sufficiently strong antibodies to two antigens, complete neutralization of either of them was possible; the remaining antibody could be identified as above. In some sera possessing strong and weak antibodies, the latter could not be identified. For instance, antiserum No. 60 was found to contain anti-A and another

TABLE 2

FREQUENCIES OF γ GLOBULIN ISO-ANTIGENS IN SIX RABBIT POPULATIONS

The lower numbers in this and the subsequent table signify the decimal fraction of the population in which the iso-antigen was present.

Antigen	*Rabbit Population*					
	Rokitnica	*Big R*	*Veterinary*	*Pathology*	*RSH*	*RSJ*
A	32 0.111	14 0.184	20 0.345	0	0	0
B	78 0.210	12 0.158	7 0.121	22 0.140	0	0
C	25 0.087	0	0	0	0	0
D	225 0.779	55 0.724	22 0.379	146 0.930	35 1.000	55 1.000
E	148 0.512	not tested				
abcd	32 0.110	8 0.105	15 0.259	4 0.025	0	0
abcde	5 0.017	not tested				
Total	289	76	58	157	35	55

'weak' antibody, which seemed not to be identical with any of those known. But in the case of serum No. 198 indirect identification of the 'weak' antibody was possible, because the reactions were identical with those of anti-A. The anti-A in this serum could not be identified directly, since after addition of an aC serum all precipitating activity was lost.

Five distinct antibodies were identified by the methods described above (two others are being analysed), and the corresponding serum antigens: A, B, C, D and E were found to be of γ globulin electrophoretic mobility.

FREQUENCIES OF γ GLOBULIN ISO-ANTIGENS AND SERUM GROUPS

Sera of 670 individual rabbits were tested with four specific iso-precipitins and 289 of them (Rokitnica) were tested also with a fifth serum, anti-E. Table 2 summarizes the results.

TABLE 3

ANTIGEN COMBINATIONS (SERUM GROUPS) OBSERVED IN SIX RABBIT POPULATIONS

No.	*Reaction with sera anti-*					*Group*	*Rabbit population*					
	A	B	C	D	E		*Rokitnica*	*Big R*	*Veterinary*	*Pathology*	*RSH*	*RSJ*
1.	+	+	+	+	+	ABCDE	0	0	0	0	0	0
2.	+	+	+	+	−	ABCDe	0					
3.	+	+	+	−	+	ABCdE	0	0	0	0	0	0
4.	+	+	+	−	−	ABCde	0					
5.	+	+	−	+	+	ABcDE	1 0.004	0	0	0	0	0
6.	+	+	−	+	−	ABcDe	7 0.024					
7.	+	+	−	−	+	ABcdE	0	3 0.040	0	0	0	0
8.	+	+	−	−	−	ABcde	0					
9.	+	−	+	+	+	AbCDE	2 0.007	0	0	0	0	0
10.	+	−	+	+	−	AbCDe	0					
11.	+	−	+	−	+	AbCdE	0	0	0	0	0	0
12.	+	−	+	−	−	AbCde	0					
13.	+	−	−	+	+	AbcDE	15 0.052	5 0.066	4 0.069	0	0	0
14.	+	−	−	+	−	AbcDe	4 0.014					
15.	+	−	−	−	+	AbcdE	3 0.010	6 0.079	16 0.276	0	0	0
16.	+	−	−	−	−	Abcde	0					
17.	−	+	+	+	+	aBCDE	2 0.007	0	0	0	0	0
18.	−	+	+	+	−	aBCDe	1 0.004					
19.	−	+	+	−	+	aBCdE	3 0.010	0	0	0	0	0
20.	−	+	+	−	−	aBCde	1 0.004					
21.	−	+	−	+	+	aBcDE	1 0.004	5 0.066	2 0.034	15 0.096	0	0
22.	−	+	−	+	−	aBcDe	42 0.145					
23.	−	+	−	−	+	aBcdE	16 0.055	4 0.052	5 0.086	7 0.045	0	0
24.	−	+	−	−	−	aBcde	4 0.014					
25.	−	−	+	+	+	abCDE	4 0.014	0	0	0	0	0
26.	−	−	+	+	−	abCDe	7 0.024					
27.	−	−	+	−	+	abCdE	1 0.004	0	0	0	0	0
28.	−	−	+	−	−	abCde	4 0.014					
29.	−	−	−	+	+	abcDE	73 0.253	45 0.592	16 0.276	131 0.834	35 1.000	55 1.000
30.	−	−	−	+	−	abcDe	66 0.228					
31.	−	−	−	−	+	abcdE	27 0.093	8 0.105	15 0.259	4 0.025	0	0
32.	−	−	−	−	−	abcde	5 0.017					
					Total		289	76	58	157	35	55

Among the six rabbit populations studied, the population Rokitnica was the most heterogeneous, possessing all the five iso-antigens. It is remarkable that the antigen C was absent in all the other populations. Antigen A was found only among Big R, Veterinary and Rokitnica rabbits.

Using antisera for four antigens, it should be possible to distinguish a maximum of sixteen serum groups (antigen combinations). So far, thirteen such groups have been demonstrated. It is interesting that populations RSH and RSJ were composed of rabbits of group abcD only.

When five antisera were employed, twenty-two out of the possible maximum of thirty-two groups were found. The group frequencies are shown in Table 3 (see also Dubiski, Skalba, Dubiska and Kelus, 1959; Dubiski and Kelus, 1961).

TABLE 4

COMPARISON OF ANTI-A AND ANTI-RGG I (NO. 38) SERA

		Reaction of antiserum No. 38		*Total*
		+	−	
Reaction of anti-A serum	+	13	0	13
	−	0	34	34
Total		13	34	47

ANALYSIS OF ANTI-RGG I AND ANTI-RGG II SERA

Dr. Dray kindly sent us anti-RGG I and anti-RGG II sera together with some sera containing the corresponding antigens RGG I and RGG II. Anti-RGG I (No. 38) serum was tested against forty-seven sera and anti-RGG II (No. 104) against eighty-eight sera.

TABLE 5

COMPARISON OF ANTI-A AND ANTI-RGG II (NO. 104) SERA

		Reaction of antiserum No. 104		*Total*
		+	−	
Reaction of anti-A serum	+	50	4	54
	−	33	1	34
Total		83	5	88

These antisera were obtained by injection of whole serum. Antiserum No. 38 contained some antibodies for non-γ-globulin antigens as well. These antibodies produced 'late' or 'weak' lines in contrast to the 'early' lines produced by anti-RGG I antibody. As can be seen from Table 4 it is very likely that anti-RGG I is identical with anti-A. Table 5 shows

the reactions of anti-RGG II in comparison with anti-A. Out of eighty-eight panel sera tested, five sera did not react with anti-RGG II. One of them (No. 394) reacted neither with anti-RGG I (anti-A) nor with anti-RGG II. It is remarkable that Dray and Young could not find a similar 'minus-minus' serum among 500 rabbit sera of several breeds.

INHERITANCE OF γ GLOBULIN ISO-ANTIGENS

Preliminary family studies, including those of six generations, were performed. The rabbit families belonged to various populations. It was established that all the antigens A, B, C, D and E are of hereditary character. These antigens could be found in offspring only when they were present in at least one of the parents. The antigens seem to form three statistically independent genetic systems: A, C and BDE (Kelus, 1960). Further studies of the family material are in progress and will be published separately.

DISCUSSION

It has been shown that at least five different iso-antigens can be distinguished in the γ globulin fraction of rabbit serum, and the analysis of antisera recently obtained suggests the existence of other iso-antigens. The RGG II antigen seems also to be distinct from those already described. In a previous paper (Dubiski, Dudziak, *et al.*, 1959) some antigens of very low frequency were mentioned. Such a great variety of serum iso-antigens might appear to be in contradiction with the results of Dray and Young (1959). In spite of many trials, they were not able to produce iso-precipitins of other than RGG I and RGG II specificity. A possible explanation is that whereas they worked with rabbits of 'pure breeds' (Dray, 1959), the animals in our experiments were of mixed breeds. It is remarkable that Dray and Young did not find in the 500 sera which they tested any in which both RGG I and RGG II antigens were absent. Our material included not only some abcde sera, but RGG I-RGG II-negative serum as well. It seems that sera of groups abcde-RGG II-negative can also exist, but must be rare. If no RGG I-RGG II-negative sera had been found, it might have been argued that genes determining antigens A (RGG I) and a (RGG II) are alleles. However, such sera do exist and therefore the inheritance of, and genetic relationship between, the γ globulin antigens appears to be more complicated, than suggested by Dray and Young.

Most antibodies to hetero-antigens are localized in the γ globulin fraction, so that the same γ globulin molecule can carry on its surface both antigenic determinants and antibody-combining sites. A preliminary immunochemical study of the γ globulin iso-antigens was carried out by Kelus, Marrack and Richards (1961). A rabbit B-γ-globulin was hydrolysed by crystalline papain (prepared by the Biochemical Department, University of Cambridge) and fractionated on carboxy-methylcellulose (Porter, 1959); three large fragments were isolated. These were examined in the Spinco analytical ultra-centrifuge and each was observed to sediment as a single peak. The measured sedimentation coefficients were in agreement with the results given by Charlwood (1959).

Goat anti-rabbit globulin serum precipitated the crystalline fraction (III). This fraction III did not react with rabbit anti-B-γ-globulin serum, whereas fractions I and II did. This suggests that the species specific antigenic sites and the iso-specific antigenic sites are located on different pieces of the γ globulin molecule. Further, three other γ globulin preparations of group D and BD were hydrolysed and fractionated using a commercial crystalline papain. This time only two fractions, I and crystalline III, were obtained.

With another sample of crystalline papain three fragments have been obtained from several samples of γ globulin. The matter is being investigated further. Fraction I of the BD-γ-globulin could be precipitated with rabbit anti-B serum, but the fractions I of both BD- and D-γ-globulin did not form precipitates with rabbit anti-D serum. The precipitate of anti-D and D-γ-globulin was much reduced by the addition of these fractions I. Thus fractions I of BD- and D-γ-globulin apparently combine with anti-D antibody although no precipitate results.

Richards (1960) found by the light scattering method that the molecular weight of fraction I was 58,000. Preliminary calculations based on sedimentation-diffusion measurements suggest molecular weights about 50,000 for both fractions I and III. In this instance, anti-bovine-serum-albumin of group D was used and only two fractions were obtained after hydrolysis.

Antigenic determinants as markers of γ globulin molecules could be of use to biochemists in their studies on protein structure. They are also of special interest for serologists; the intra-species antigenic differentiation of γ globulins might be of importance in transplantation immunity.

Use could be made of serum iso-antigens as hereditary markers in genetic experiments. It is to be stressed that samples of sera as panel antigens can be stored frozen for a long time — a highly important fact for biological standardization.

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