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Serum Groups in Rabbits

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Summary. By immunizing rabbits with bacteria agglutinated by corresponding immune rabbit sera group iso-precipitins were obtained. Using the iso-precipitins thus obtained the rabbit sera could be divided into two serum groups: D (a+) and D (a−); for grouping the gel diffusion technique was applied.

The serum groups are heritable; probably group D (a+) is inherited as a dominant character. D (a+) mothers can transfer D^a protein non-genetically. The substance thus obtained is then gradually eliminated from the bloodstream of the young animal. D^a antigen has γ-globulin electrophoretic mobility as shown by means of immunoelectrophoresis. The incidence of group D (a+) in 92 unrelated rabbits was 27·2 per cent.

The authors discuss the relationship between iso-precipitins and anti-antibodies and the practical significance of rabbit serum groups analogous to human Gm serum groups.

INTRODUCTION

In recent years the attention of serologists has been attracted by the discovery of the serological differences of human serum proteins. Grubb and Laurell (1956) described the existence of two human serum groups: Gm (a+) and Gm (a−). As the reagent for determination of these groups they used selected sera from people suffering from rheumatoid arthritis containing the factor which agglutinates human red cells sensitized with selected human anti-Rh sera. This agglutinating factor was neutralized by the addition of normal human serum of Gm (a+) group. The addition of human Gm (a−) serum did not have any effect on the agglutination of sensitized red cells.

The use of rheumatoid arthritis sera as reagents for determination of serum groups connects this problem with that of so-called 'anti-antibodies'. This term was proposed by Milgrom, Dubiski and Woźniczko (1956 a,b,c) to denote the factor contained in the sera of some normal people which agglutinates red cells sensitized with human anti-Rh sera. This factor, according to Milgrom *et al.*, is an antibody for serologically denatured immune globulins, since it reacts with human immune globulins only after previous combination of these antibodies with the antigen.

Properties similar to those of anti-antibodies are displayed by the long-known Agglutination Activating Factor (AAF) present in some rheumatoid arthritis sera (Meyer, 1922; Waaler, 1940; Rose *et al.*, 1948). This factor agglutinates sheep red cells sensitized with rabbit anti-sheep red cell amboceptor. Grubb (1956) showed that some rheumatoid arthritis sera also contain a factor agglutinating red cells sensitized with human anti-Rh sera, but this reaction can be inhibited by the addition of unchanged sera from some persons, later defined by Grubb and Laurell (1956) as persons belonging to the Gm (a+) group. The agglutinating factor contained in a second type of sera was not neutralized by the addition of Gm (a+) sera; this type was called by Grubb 'non-rheumatoid arthritis

type' and was most probably comparable to the sera described by Milgrom *et al.* (anti-antibody type).

Two factors similar to those described above were produced experimentally in rabbits by Milgrom and Dubiski (1957) and by Dubiski (1958). The rabbits were injected with bacteria agglutinated by rabbit immune sera. The serum obtained from rabbits immunized in this manner agglutinated sheep red cells sensitized with rabbit anti-sheep-red-cell amboceptor. As Dubiski showed, two types could be demonstrated among the sera studied. The first type of sera was not neutralized by any of the normal rabbit sera and reacted only with rabbit immune globulins modified by combination with antigen. This type was similar to anti-antibodies of human sera described by Milgrom *et al.* or Grubb's 'non-rheumatoid arthritis' type of sera. The second type was neutralized by unchanged sera of certain rabbits and was similar to sera described as rheumatoid arthritis type (Grubb). Dubiski supposed that the ability to inhibit the reaction of these sera was connected with the presence of rabbit serum groups analogous to those described by Grubb and Laurell and the sera may be supposed to contain iso-precipitins.

The neutralization technique, however, did not give clear-cut results and the rabbit sera could not be grouped by means of this method. In the present paper an attempt was made to apply the gel diffusion technique for examination of rabbit sera obtained by injecting immune complexes and for studying the serological differences between rabbit sera.

MATERIALS AND METHODS

1. PRODUCTION OF ISO-PRECIPITIN SERA

For the production of iso-precipitin sera a method of iso-stimulation as described by Dubiski (1958) was used. The rabbits were injected with immune complexes formed by bacteria with the corresponding immune antibacterial rabbit serum. These antibacterial sera were produced by injecting another group of rabbits with heat killed bacterial suspensions of the following species: *Proteus X* 19, *Salmonella typhi murium*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Serratia marcescens*. To prepare the inoculum for injection 1 ml. of antibacterial serum was mixed with 2 drops of 50 per cent suspension of corresponding bacteria. The mixture was then incubated for 60 minutes at 37° C., then washed three times, re-suspended in 2 ml. of saline and injected intravenously. Each rabbit during the whole period of stimulation was immunized with bacteria agglutinated by antiserum made by one and the same donor. The injections were made twice a week. Rabbits received ten to fifteen injections.

2. GEL DIFFUSION TECHNIQUE (OUCHTERLONY 1948)

Agar for the tests was prepared as described by Grabar and Williams (1955). One and a half per cent solution of agar in 0·15 M NaCl buffered with 0·02 M phosphate buffer, *p*H 7·2–7·4 was used. Thiomersalate 1 : 10,000 was added as a preservative.

Six basins were formed on the circumference and one in the centre of an agar plate. Usually the central basin was filled with antiserum, and the peripheral with the sera being tested (antigens) as shown in Fig. 1. Antisera were used undiluted or diluted 1 : 2, antigens were diluted 1 : 3–1 : 5. The results were noted after five days and the readings were re-checked after seven and fourteen days.

3. AGGLUTINATION OF SENSITIZED SHEEP RED CELLS

Sheep red cells were mixed with sub-agglutinating dilution of rabbit anti-sheep-red-cell haemolytic serum, then incubated at room temperature at 19° for 30 min. and washed three times. The agglutination tests were performed on slides. To 1 drop of 3 per cent suspension of sensitized sheep red cells 1 drop of the serum studied was added; the results were read after 30 min. at room temperature.

4. NEUTRALIZATION OF THE SERA

To 1 part of the appropriate dilution of rabbit immune serum studied 1 part of 1 : 10 dilution of neutralizing serum was added. The mixture was left for 30 min. at room temperature and then tested using the above described agglutination technique.

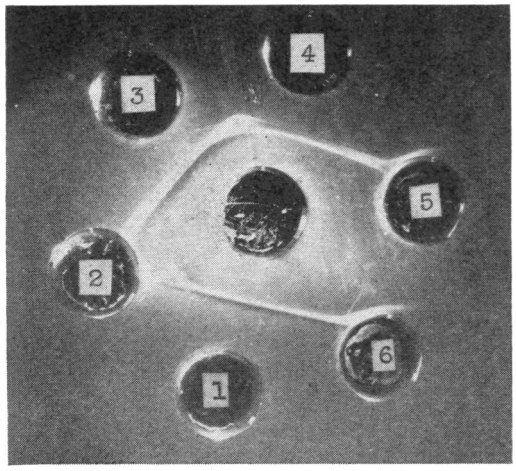

FIG. 1. Determination of serum groups in rabbit by means of gel precipitation test. In the central basin—anti-D^a serum, in circumferential basins—the tested sera (antigens). Results: sera Nos. 1, 3, 4 are of group D (a+), sera Nos. 2, 5, 6 are of group D (a−).

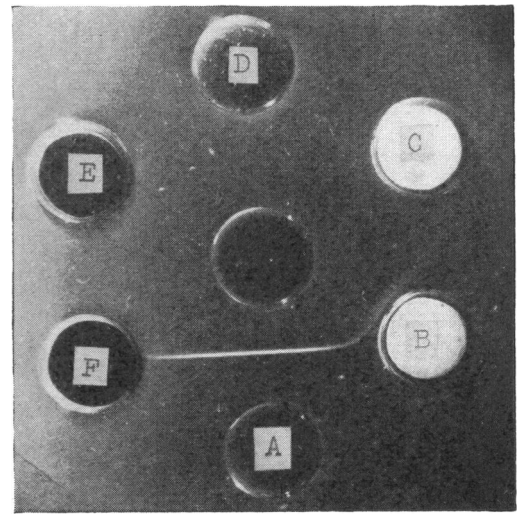

FIG. 2. Reaction of the antiserum No. 574 ('anti-antibody' type) with the homologous serum antigen No. 560. The central basin was filled with antiserum 574, the basin 'A' with unchanged homologous serum (antigen), basin 'B' with a suspension of bacteria (*Proteus X* 19) agglutinated by the homologous serum and then washed. The basin 'C' was filled with unchanged *Proteus X* 19 suspension, and basins 'D', 'E', 'F' with 3 random rabbit sera. The homologous serum produced a precipitation line when unchanged as well as when dissociated from the immune complex.

RESULTS

1. RABBIT SERUM GROUPS—NOMENCLATURE

Those immune sera obtained by injecting immune complexes which could be neutralized by some normal rabbit sera in the test with sensitized sheep red cells were also active in the gel precipitation test. Conversely rabbit sera precipitated by these immune sera were also capable of inhibiting the agglutination of sensitized sheep cells by these sera.

This made it possible to divide the tested rabbit sera into two groups: D (a+) and D (a−). To the D (a+) group belonged those sera which reacted in gel precipitation test and displayed neturalizing properties, while group D (a−) consisted of sera that did not possess these properties.

The above nomenclature was proposed on the basis of analogy with the generally accepted principles of nomenclature for red cell group factors. The letter 'D' was used as an abbreviation for the word 'denaturation', since the iso-precipitins were produced by injections of immune complexes, in which the stimulating antigen (immune globulin) was supposed to be denatured on combination with antigen.

2. IMMUNE SERA

The table below (Table 1) presents the relationship between the method employed to obtain the immune sera and the properties of these sera.

As can be seen from Table 1, the properties of the immune sera obtained depend above all on the serum used for stimulation. When the serum used for stimulation is taken from

TABLE 1

PROPERTIES OF IMMUNE SERA ON RELATIONSHIP TO THE MANNER IN WHICH THEY WERE OBTAINED

		Serum type					
		anti-D_a isoprecipitin			*anti-antibody*		
Rabbit No.		178 D (a−)	176 D (a+)	452 D (a−)	574 D (a+)	542 D (a−)	566 D (a−)
Stimulated with serum		569 D (a+)		453 D (a+) 939 D (a+)	560 D (a−)		557 D (a−)
Bacteria used for preparing immune complexes		*S. typhi murium*		*Proteus X* 19			*E. coli*
Reaction with sheep red cells sensitized by amboceptor of group	D (a−)	+	+	+	+	+	+
	D (a+)	+	+	+	+	+	+
Neutralization by unchanged serum of group	D (a−)	−	−	−	−	−	−
	D (a+)	+	+	+	−	−	−
Precipitation in gel with serum of group	D (a−)	−	−	−	−	−	−
	D (a+)	+	+	+	−	−	−
Reaction with serum used for stimulation (homologous serum)	neutralization	+	+	+	+	+	+
	precipitation in gel	+	+	+	+	+	+

rabbits belonging to the group D (a+), then anti-D^a iso-precipitin is produced. In the case when serum of group D (a−) is employed for stimulation, the resulting anti-antibody reacts with antibodies combined with antigens when tested with sensitized sheep red cells and also with unchanged homologous serum (i.e. serum used for the stimulation) in a gel diffusion test (Fig. 2). Comment is made later on serum 176.

Table 1 gives only those sera which were obtained by stimulation with agglutinated Gram-negative bacteria (*S. typhi murium*, *E. coli* and *Proteus X* 19). Stimulation was also carried out with agglutinated *B. subtilis* and *Staph. aureus*, but the results were much poorer than when Gram-negative bacteria were employed. The rabbit stimulated with *Serratia marcescens* died before the antiserum was sufficiently strong. Prolonged immunization of rabbits with human red cell stromata agglutinated with rabbit serum was without effect. The vehicle, bacteria or stromata, used for stimulation acted probably not only as an adsorbent and denaturating factor but also as a kind of adjuvant named by Sachs (1928) 'Schlepper'. Globulins of rabbit sera, even after serological 'denaturation,' are evidently not sufficiently antigenic by themselves to induce the production of antibodies and require the presence of a strong antigen acting as a 'Schlepper'.

3. COMPARISON OF THE REACTIONS OF THE SERA

Samples of 126 rabbit sera were tested simultaneously with sera 452 and 178 (for the characteristics of these sera see Table 1). The correlation between the reactions of these sera was not absolutely complete. The following table lists the tests with sera 178 and 452 (Table 2).

TABLE 2

RESULTS OF GEL PRECIPITATION TESTS USING ANTISERA 178 AND 452

		Reaction of Serum 178		Total
		+	−	
Reaction of Serum 452	+	35	4	39
	−	0	87	87
Total		35	91	126

As can be seen from Table 2, serum 452 contains, apart from antibodies that serum 178 also contains, antibodies which the latter does not have. This indicates the possibility of there being further group antigens of rabbit serum in addition to antigen D^a. With sera 178 and 176 the correlation was even closer, but again not 100 per cent.

In further experiments serum 178 was taken as anti-D^a test serum and the reactions of this serum were regarded as decisive.

4. INHERITANCE OF SERUM GROUPS

Gel diffusion tests were made on 61 rabbits from 11 litters. The following table presents the experimental material (Table 3).

The experiments demonstrate without any doubt the inheritable character of the rabbit serum groups. Group D (a+) is probably inherited as a dominant character. These observations, however, require confirmation on more extensive material.

5. NON-GENETIC TRANSFER OF D^a ANTIGEN FROM MOTHER TO INFANT

In determination of serum groups in young rabbits it was found that in cases when the mother belonged to group D (a+) usually all of her offspring belonged to the same group.

TABLE 3

SERUM GROUPS OF OFFSPRING IN RELATIONSHIP WITH THE SERUM GROUPS OF THE PARENTS*

Parents	*Number of litters*	*Offspring*		
		D (a+)	*D (a−)*	*Total*
D (a+) × D (a+) ..	1	1	1	2
D (a+) × D (a−) ..	5	13	11	24
D (a−) × D (a−) ..	5	0	35	35
Total	11	14	47	61

* Sera of the young rabbits were taken from animals of at least 8 weeks of age.

Control tests performed some time later showed that part of the rabbits grouped previously as D (a+) subsequently reacted as D (a−). The Table 4 presents the results of tests made on one of the litters under observation.

The most probable explanation for this phenomenon is given by assuming a non-genetic transfer of the antigen D^a from the mother's to the offspring's circulation. The serum group of rabbits Nos. 2-5 (see Table 4) was undoubtedly genetically determined as D (a+), which was confirmed by control tests made several months after their birth.

TABLE 4

THE BEHAVIOUR OF SERUM GROUPS IN THE OFFSPRING OF FATHER D (a−) AND MOTHER D (a+) IN RELATIONSHIP TO THE AGE (REACTION WITH ANTI-D^a)

Age in weeks	*Rabbit No.*						
	1	2	3	4	5	6	7
2½	+	+	+	+	+	+	+
8	±?	+	+	+	+	−	died
18	−	+	+	+	+	−	
24	−	+	+	+	+	−	

The serum group of rabbits Nos. 1 and 6, on the other hand, was determined genetically as D (a−). These rabbits, however, temporarily displayed a reaction typical of D (a+), since maternal D^a protein has presumably reached the foetus *in utero* or later via the colostrum. Periodic control tests showed gradual elimination of this antigen.

6. IMMUNOELECTROPHORETIC ANALYSIS

Professor J. R. Marrack very kindly examined for the authors the D (a+)—anti-D^a precipitin system by means of immunoelectrophoresis. It was found that the D^a antigen is of γ-globulin mobility (Fig. 3).

7. FREQUENCY OF D (a+)

Tests were made on 92 serum samples of unrelated rabbits. The D (a+) group was found in 25 rabbits. The incidence of D (a+), therefore is 27·2 per cent in this population.

DISCUSSION

On the basis of the experiments carried out it is possible to characterize two types of immune sera obtained by iso-stimulation: 'anti-antibody' (i.e. antibody for antibody combined with antigen) and iso-precipitin. The properties of both types of sera have been compared in Table 1. It should be stressed that the neutralization of iso-precipitin sera, as examined with sensitized sheep red cells, by unchanged D (a+) sera did not always give clear-cut results. Iso-precipitins of some sera are not neutralized by unchanged serum, but are much more easily absorbed by immune complexes composed of antigen and immune serum of D (a+) group. As a result Milgrom and Dubiski (1957) believed that the 'anti-antibodies' which they studied exhibited some 'specificity'. They found that the 'anti-antibody' produced by stimulation with agglutinated *E. coli* could be absorbed by sheep red cell stromata, agglutinated by rabbit antibody, as well as by the homologous (*E. coli*) bacteria agglutinated by *E. coli* antiserum, but not by agglutinated *Proteus X* 19. The 'anti-antibody' produced by stimulation with agglutinated *Proteus X* 19 could be absorbed by agglutinated sheep red cell stromata as well as by homologous (*Proteus X* 19) bacteria agglutinated by Proteus antiserum, but not by agglutinated *E. coli*. In these

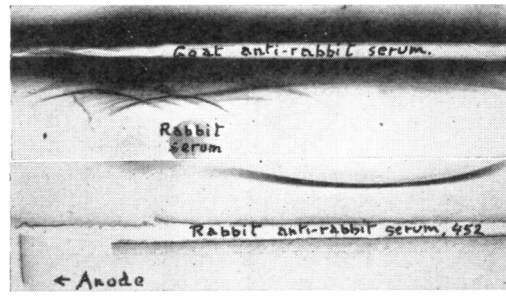

FIG. 3. Immunoelectrophoresis of the D (a+) serum. The pattern was developed by goat anti-rabbit whole serum precipitin (upper trench) and by the iso-precipitin serum No. 452 (lower trench).

authors' experiments the unchanged antibacterial serum did not exhibit inhibitory properties. Probably, however, the sera observed were not the pure 'anti-antibody' type, but contained iso-precipitins also. These sera were obtained by stimulation with pooled antibacterial sera and hence may have had iso-precipitin specificities other than anti-D^a; this may explain the results obtained by Milgrom and Dubiski. More detailed considerations on the subject of the properties of the 'anti-antibody' type of sera will be published in another paper.

Anti-D^a iso-precipitins arise as the result of stimulation of rabbit with immune complexes consisting of bacteria and group D (a+) rabbit antibodies. An unexpected phenomenon was the production of anti-D^a iso-precipitins by 3 rabbits which were grouped as D (a+). One of these 3 rabbits is listed in Table 1 as an example (rabbit No. 176). It is not easy to offer an explanation of these findings, unless it lies in further individual antigenic differences of the sera of the donor and the immunized animal.

Another interesting phenomenon was the formation of precipitins for homologous serum (i.e. the serum used for stimulation). Sera of the 'anti-antibody' type, showing hardly any iso-precipitin reaction against the sera of 20 random rabbits, nevertheless always gave the reaction in gel with the homologous serum. This phenomenon can be

explained on the assumption that the serum of every rabbit possesses, apart from the D^a globulin specificity, other group and/or individual antigenic specificity. Upon stimulation with D (a+) serum these antigenic factors may be overshadowed by the D^a specificity.

Another question is the significance of rabbit serum groups as an experimental model of analogous human groups. The gel precipitation test with the use of strong antisera is a convenient and sure method for determination of serum groups (see Fig. 1). The application of such technique to studies of human Gm groups would undoubtedly constitute a great stride forward in this field; experiments along this line are now being conducted in this Institute, and the detailed results will be published at a later date.

The incomplete correlation between the reactions of anti-D^a iso-precipitins give reason to believe that by appropriate choice of donor and recipient in stimulation it will be possible to show further serum group (or subgroup) factors apart from the D^a. It is not unlikely that it will also be possible to show further serum group factors in man if the appropriate method were applied.*

The finding of non-genetic transfer of D^a protein from mother to her offspring and the gradual elimination of protein so transferred confirms and supplements the observations of Brönnestam and Nilsson (1957), who supposed that a similar mechanism of transferring Gm substance exists in human beings.

The phenomenon of the existence of serum groups probably is not restricted to man and rabbit. The authors succeeded in production of iso-precipitins in guinea pigs; more detailed data on these experiments will be published at a later date.

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* It would be most important to work out a safe and sure method for production of strong iso-precipitins in men.

NOTE

After this paper was submitted the authors' attention was drawn to the work of Oudin*. Oudin injected rabbits with immune precipitates formed by rabbit immune serum and corresponding antigen. These precipitates were mixed with Freund's adjuvant. Such immunized rabbits produced iso-precipitins which were active in gel diffusion tests against the homologous unchanged serum and sera of some other rabbits. It seems very plausible, if not certain that the D^a serum group 'system' described in this paper is one of the possible manifestations of l' 'allotypie' of Oudin.

Also, in a paper that appeared (*J. Immunology*, 1958, *81*, 142) after this paper had been accepted for publication, Dray and Young showed that iso-precipitins could be induced by injection of rabbit sera together with paraffin-oil type adjuvants and that 90 rabbits tested could be grouped into 13 groups on the basis of formation or non-formation of precipitin bands in gel diffusion tubes with 6 rabbit antisera.

* Oudin, M. J. (1956). 'Réaction de précipitation spécifique entre des sérums d'animaux de même espece.' *Comp. rend. Acad. Sci., Paris*, **242**, 2489–90.

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