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Inhibition of the Anti-Globulin Reaction by Human Immunoglobulin G and its Fragments

P. J. Grob, H. C. Isliker and T. Webb

Institut de Biochemie and the W.H.O. International Reference Centre for Immunoglobulins, Université de Lausanne, Switzerland

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Summary. The specificities of antibodies involved in the agglutination of anti-D sensitized human red cells was studied using IgG and its fragments Fc and Fab as inhibitors. It was shown that commercial anti-globulin sera and specific sheep anti-IgG serum react with the antigenic groups common to IgG and Fc. Anti-Fab serum on the other hand agglutinated anti-D sensitized red cells by reacting with the antigenic groups common to IgG and Fab. This result is interpreted to mean that antibodies reacting with Fab have different specificities depending on whether IgG or Fab was used as antigen.

INTRODUCTION

Anti-globulin reagents are usually produced by immunization with a crude globulin fraction of normal human serum and therefore contain antibodies of several different specifities reacting with many of the globulins of normal human serum. However the antibodies responsible for the agglutination of anti-D sensitized red cells are those that react with the anti-D antibodies which sensitize the cells. In general these anti-D antibodies are mostly of the IgG-type (Dodd and Wilkinson, 1964). Coombs and Mourant (1947), and Dacie (1951) demonstrated that the components in anti-globulin sera reacting with red cells sensitized with Rh antibodies were most probably anti-IgG antibodies.

Anti-IgG antibodies may react with many different antigenic determinants of the IgG molecule. These determinants can be partially separated by the isolation of fragments and chains of the molecule. Fudenberg and Franklin (1965) demonstrated that anti-γ chain (H or A chain of IgG) antibodies gave agglutinating titres comparable to those given by anti-globulin sera. Absorption of anti-globulin sera with partially purified fragments Fab and Fc showed that the reaction with anti-D sensitized cells was more sensitive to absorption with Fc than with Fab (Rawson and Abelson, 1964). In the present study we have confirmed and extended these results; highly purified IgG and its fragments Fab and Fc have been used to study the quantitative inhibition of various anti-globulin sera agglutinating anti-D sensitized human red cells. In addition, we have studied the agglutination of these red cells by an anti-Fab serum.

MATERIAL AND METHODS

Antigens

Two preparations of human IgG prepared as described in detail elsewhere (Grob, Frommel, Masouredis and Isliker, unpublished) were used: (a) human IgG of the Central

Laboratory of the Swiss Red Cross (lot 6379, Cohn fraction II, lyophilized), which was further purified by adsorption of impurities on to DEAE-cellulose (equilibrated at pH 7·4 and ionic strength 0·01), and (b) human IgG prepared from the serum of a normal donor (blood group O, Rh positive). The serum was centrifuged in a Spinco Model L 50 ultracentrifuge at 135,000 ***g*** for 16 hours. The upper third in the tube was then removed by pipetting to eliminate the lipoproteins. The remaining solution was decanted in order to remove the pellet and finally passed twice through a column of DEAE-cellulose (equilibrated at pH 8 and ionic strength of 0·01).

The human IgG (preparation (a) above) was digested by papain under the following conditions (Porter, 1959): 400 mg of purified IgG were incubated for 17 hours with papain (1·5 mg papain per 100 mg IgG) in the presence of cysteine and EDTA.

The digested material was separated, according to the technique of Franklin (1960), using CM-cellulose equilibrated with phosphate buffer (0·01 M, pH 7·6) for the elution of fraction I and the same phosphate buffer containing 0·4 M NaCl for the elution of fraction II. Fraction I was further separated into fractions A and B on DEAE-cellulose using phosphate buffers of 0·01 M, pH 7·6 and 0·03 M, pH 7·6 respectively. Fraction II was further separated on DEAE-cellulose into fractions C and D by the same two phosphate buffers (0·01 M, pH 7·6 and 0·03 M, pH 7·6). Fractions A, B and C were each further purified by passage through columns (2×90 cm) of Sephadex G-200 (1 M NaCl, 0·05 M phosphate, pH 8·2) in order to remove trace amounts of non-digested IgG and degradation products smaller than Fab and Fc. As shown below, Fab corresponds to fractions A and C, and Fc to Fraction B.

Antisera

Anti-globulin sera. Three different commercial preparations were used: (a) high titred rabbit anti-human globulin (lot R 7232 prepared by the Central Laboratory of the Swiss Red Cross, Berne); (b) rabbit anti-human serum (lot 7281 Ortho, Raritan, New Jersey); and (c) high titred rabbit anti-human globulin (lot 6349 from the Institute Dr H. Molter, Heidelberg).

A sheep anti-IgG was prepared by intramuscular injection of 2 mg highly purified IgG in complete Freund's adjuvant, repeated twice at intervals of 15 days. Before use, the antiserum was absorbed three times with an equal volume of packed washed human red cells (group O, Rh positive). The specific precipitating antibody concentration was determined by the precipitin reaction as described by Heidelberger and Kendall (1929).

Other antisera. A rabbit anti-Fab was prepared by two injections of 2 mg highly purified fragment Fab at an interval of 15 days. Before use the antiserum was absorbed with human red cells as described above.

For immunoelectrophoresis a mixture of two equine anti-human sera (Institut Pasteur, Nos. 419 and 223) was used.

Agglutinating system

Sensitization of red cells: the human red cells (group O, Rh positive) from blood bank donors were stored in Alsever's solution for 3–14 days and washed three times with phosphate buffered saline pH 7·3 and ionic strength 0·15 before use. Ten millilitres of a 2 per cent red cell suspension were incubated with 1 or 2 ml of an incomplete anti-D serum (anti-D test serum of the Central Laboratory of the Swiss Red Cross, Berne, lot 625). After three washings with phosphate buffered saline, the anti-globulin test was

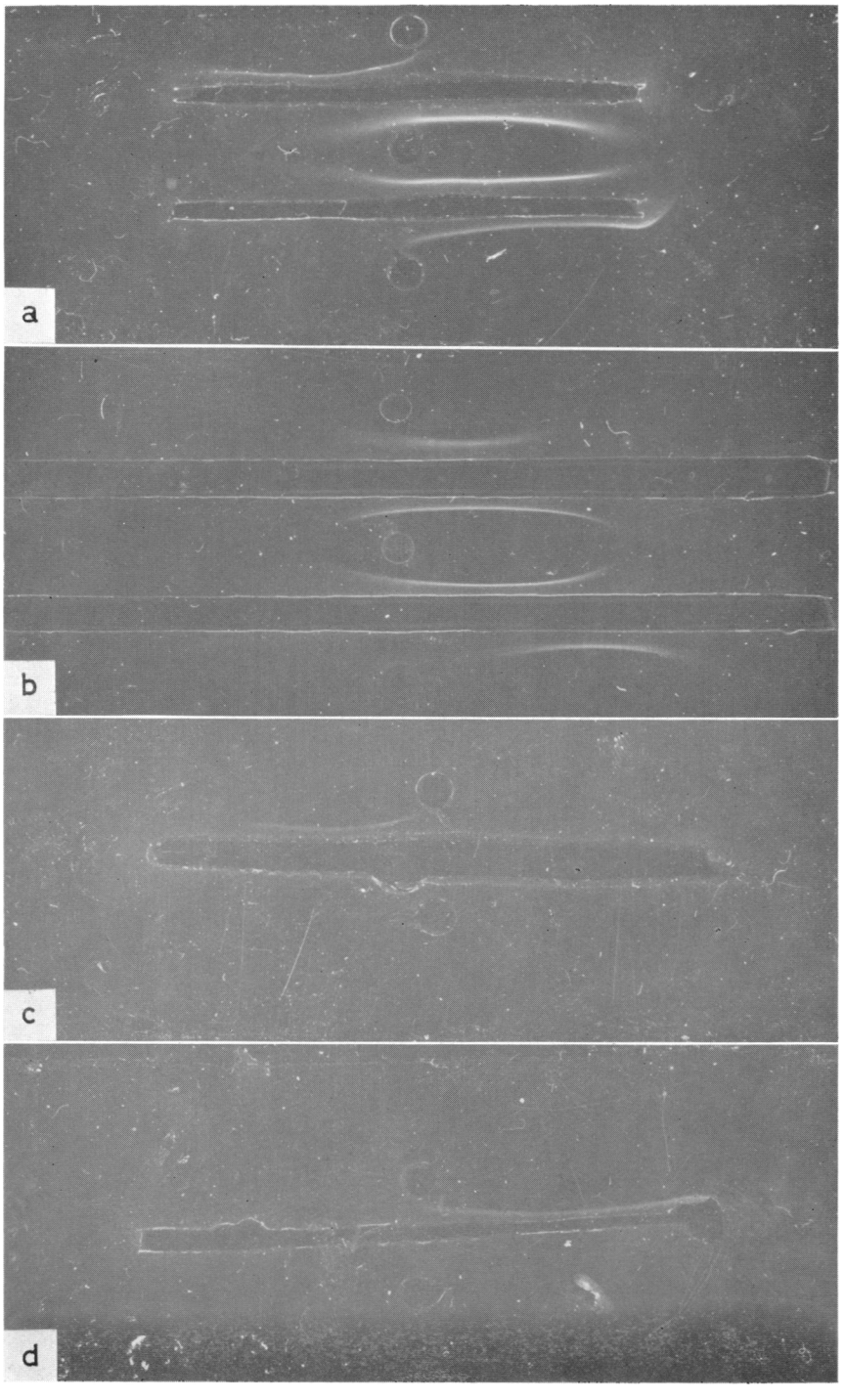

FIG. 1. Immunoelectrophoretic patterns of IgG and its fragments Fc and Fab: (a) Pattern given by (top) Fc, (middle) IgG and (bottom) Fab revealed by sheep anti-IgG; (b) pattern given by (top) Fab (fraction A), (middle) IgG and (bottom) Fab (fraction C) revealed by rabbit anti-Fab; (c) pattern given by (top) Fc, (bottom) Fab revealed by sheep anti-IgG absorbed with Fab; and (d) pattern given by (top) Fab and (bottom) Fc revealed by sheep anti-IgG absorbed with Fc. Concentrations: IgG 5 mg/ml, Fab and Fc 4 mg/ml.

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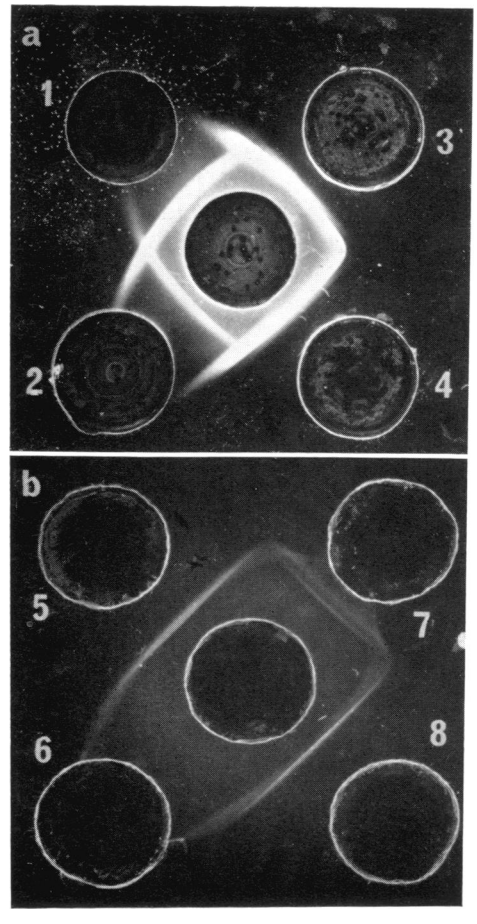

FIG. 2. Precipitin pattern of IgG and its fragments Fab and Fc in Ouchterlony plates: (a) Fab (1). Fc (2) and IgG (3 and 4) revealed by sheep anti-IgG; and (b) Fab (5 and 8), Fc (6) and IgG (7) revealed by rabbit anti-Fab. Concentrations: IgG 5 mg/ml, Fab and Fc 4 mg/ml.

performed according to Dunsford and Grant (1959); 0·1 ml of a 2 per cent suspension of anti-D sensitized red cells were mixed with 0·1 ml of anti-globulin serum or anti-globulin serum plus inhibitor. After 30 minutes at room temperature the tubes were centrifuged (1000 rev/min, for 1 minute) and the agglutination titre was read. The titre was expressed as the reciprocal of the last dilution of the anti-globulin serum which gave a macroscopic clumping of the red cells.

Inhibition studies

Anti-globulin serum 0·1 ml, in double dilutions were incubated with 0·1 ml of inhibitor (IgG, Fab or Fc) for 30 minutes at room temperature. The anti-globulin test was subsequently performed as described above.

Other techniques

Immunoelectrophoresis was done with the micromethod according to Scheidegger (1955) with gelose or agarose 2 per cent in a Veronal buffer 0·025 M, pH 8·2. The same material was used to perform the double immunodiffusion technique of Ouchterlony (1953). Ultracentrifugation was carried out in a Spinco Model E Ultracentrifuge at 59,720 rev/min at 25° using a protein concentration of 8 mg/ml in a 0·015 M phosphate–saline buffer.

RESULTS

PURITY OF IgG AND FRAGMENTS

The immunoelectrophoretic analysis of IgG at a concentration of 8 mg/ml using a potent anti-human serum, revealed a single precipitin line. The immunoelectrophoretic analysis of fractions A and C revealed single components having slightly different mobilities but each corresponded to Fab (Fig. 1b). These fractions were, therefore, mixed and used as Fab in all subsequent experiments. The electrophoretic mobility of Fab was less than that of IgG while that of fragment B (Fc) was greater (Fig. 1a). By the double diffusion technique in agar gel, the precipitin lines of Fc and Fab crossed completely without trace of a common line when revealed with the sheep anti-IgG (Fig. 2a), while Fc (8 mg/ml) gave no reaction with anti-Fab (Fig. 2b). The ultracentrifugation of IgG revealed one symmetrical peak with a sedimentation coefficient of 6·6 S, while Fab and Fc each showed a constituent with a sedimentation coefficient of 3·5 S.

PROPERTIES OF THE ANTISERA

All three commercial preparations of rabbit anti-human globulin and the purified sheep anti-IgG showed precipitin lines in immunoelectrophoresis when reacted with Fab and Fc (Fig. 1a). These precipitin lines given by the fragments and the sheep anti-IgG could be separately and specifically inhibited by adsorption with the appropriate fragment (Fig. 1c and d). The sheep anti-IgG serum contained 8·5 mg/ml of antibody precipitable by IgG of which 2·2 mg/ml was precipitated by Fab.

The rabbit anti-sera even diluted 1 : 10 gave a precipitin line in double immunodiffusion with Fab (1 mg/ml of Fab) while even at a concentration of 4 mg/ml the Fc gave neither a precipitin line with anti-Fab nor was there a deviation of the precipitin line between Fab and anti-Fab when revealed in proximity of Fc (Fig. 2b).

INHIBITION OF ANTI-GLOBULIN REACTION BY IgG AND ITS FRAGMENTS

The results in Table 1 show that the different anti-globulin sera could be completely inhibited by highly purified IgG. It was also shown that Fc on a molar basis was only slightly less potent as an inhibitor in this reaction. Fab however showed very little activity in inhibiting these anti-globulin sera. Table 2 gives the results obtained when the inhibitory

TABLE 1

TITRES OF DIFFERENT ANTI-GLOBULIN SERA IN THE PRESENCE OR ABSENCE OF IgG AND ITS FRAGMENTS Fc AND Fab

Anti-globulin serum	Inhibitor			
	IgG 270 μg	Fc 90 μg	Fab 1700 μg	Control NaCl
Rabbit anti-human serum (Ortho)	0*	0	128	256
Rabbit anti-human globulin serum (Swiss Red Cross)	0	4	512	1024
Rabbit anti-human globulin serum (Institute Dr Molter)	0	4	256	1024
Sheep anti-human IgG (highly purified)	2	4	512	2084

* 0, No agglutination with undiluted antisera.

TABLE 2

INHIBITION OF THE AGGLUTINATING ACTIVITY OF AN ANTI-IgG SERUM BY IgG AND ITS FRAGMENTS Fc AND Fab*

Sheep anti-IgG dilution	Antibody (μg)	Inhibitor (μg)														
		IgG					Fc					Fab					Control
		86	43	21·5	10·8	2·7	33	16·5	8·3	4·1	1·0	170	60	30	15	3·8	NaCl
8	106	−	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+
16	53	−	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+
32	26·5	−	−	−	−	+	−	−	−	+	+	+	+	+	+	+	+
64	13·3	−	−	−	−	+	−	−	−	+	+	+	+	+	+	+	+
128	6·6	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+
256	3·3	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
512	1·7	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
1024	0·8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+, Distinct agglutination; −, no agglutination.

* 0·1 ml of diluted anti-IgG (double dilutions) incubated with 0·1 ml of inhibitor at different concentrations for 30 minutes at 20°; 0·1 ml of this mixture was then reacted with 0·1 ml of a 2 per cent suspension of anti-D sensitized human red cells.

effect on the sheep anti-IgG of various quantities of IgG, Fab and Fc was measured. For the purpose of comparison on the basis of moles of any given antigenic group, 1 μg of IgG corresponds to 0·33 μg Fc and 0·66 μg Fab. It is clear from the table that in this particular experiment the limit of positive reaction was given by approximately 2 μg of anti-IgG and that the quantity of IgG required for inhibition was less than 1 μg per μg of antibody present. On a molar basis Fc was almost as efficient an inhibitor of the reaction as IgG.

TABLE 3

INHIBITION OF THE AGGLUTINATING ACTIVITY OF AN ANTI-Fab SERUM BY IgG AND ITS FRAGMENTS Fc AND Fab*

Rabbit anti-Fab dilution	Antibody (μg)	Inhibitor (μg)																	
		IgG						Fc						Fab					Control
		40	20	10	5	2·5	1·25	140	70	35	18	9	4·5	40	20	10	5	2·5	1·25	NaCl
4	70	−	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
8	35	−	−	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+
16	17·5	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+
32	8·8	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+
64	4·4	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−	+	+
128	2·2	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	+
256	1·1	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	+
512	0·5	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+
1024	0·25	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+
2048	0·13	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+, Distinct agglutination; −, no agglutination.

* 0·1 ml of diluted anti-Fab (double dilutions) incubated with 0·1 ml inhibitor at different concentrations for 30 minutes at 20°; 0·1 ml of this mixture is then reacted with 0·1 ml of a 2 per cent suspension of anti-D sensitized human red cells

Fab on the other hand only partially inhibited the reaction (three dilutions) even when compared at a concentration giving a four-fold molar excess over IgG.

The anti-Fab serum acted as a potent (1/1000) agglutinating serum in the anti-globulin test. Table 3 shows the results obtained, when this antiserum was inhibited by IgG and

TABLE 4

INHIBITION OF THE AGGLUTINATING ACTIVITY OF A MIXTURE OF ANTI-IgG AND ANTI-Fab BY IgG AND ITS FRAGMENTS Fc AND Fab*

Dilution of antiserum†	Quantity of inhibitor (μg)						
	IgG		Fc		Fab		Control
	126	20	58	8	40	10	NaCl
4	+	+	+	+	+	+	+
8	−	+	+	+	+	+	+
16	−	+	+	+	+	+	+
32	−	−	+	+	+	+	+
64	−	−	+	+	+	+	+
128	−	−	+	+	+	+	+
256	−	−	−	+	+	+	+
512	−	−	−	−	+	+	+
1024	−	−	−	−	+	+	+
2048	−	−	−	−	+	+	+
4096	−	−	−	−	−	−	+
8192	−	−	−	−	−	−	+
16384	−	−	−	−	−	−	−

* 0·1 ml of this mixed antiserum incubated with 0·1 ml of inhibitor for 30 minutes at 20°. To 0·1 ml of this mixture was added 0·1 of a 2 per cent suspension of anti-D sensitized human red cells.

† This antiserum was made up of equal parts of sheep anti-IgG (8·6 mg/ml antibody) and rabbit anti-Fab (2·8 mg/ml antibody).

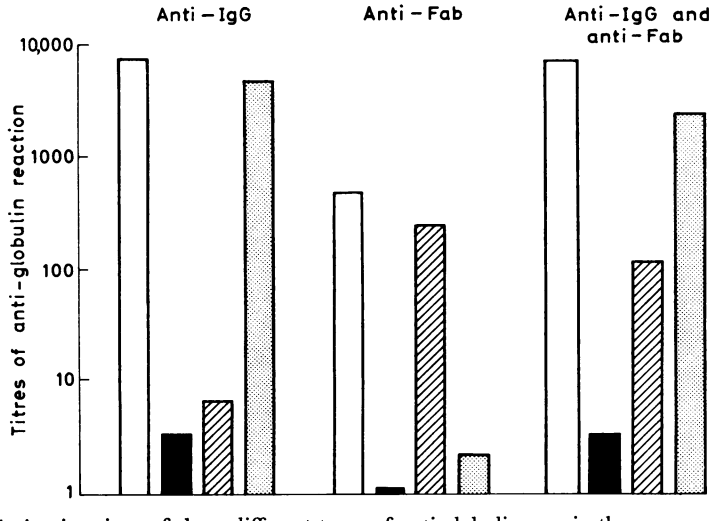

FIG. 3. Agglutination titres of three different types of anti-globulin sera in the presence of equimolar concentrations of IgG, Fc and Fab. Unshaded, control NaCl; black, IgG (126 μg); cross-hatched, Fc (40 μg); stipple, Fab (80 μg).

its fragments Fc and Fab. In this case IgG and Fab were equally very effective inhibitors of the reaction. Fc on the other hand when used at a six-fold molar excess over IgG did not inhibit the reaction more than three dilutions.

A third type of anti-globulin serum was prepared artificially by mixing the anti-IgG and the anti-Fab in equal proportions. In this case only the IgG was an effective inhibitor of the reaction. Fab and Fc used at molar excess over IgG only partially inhibited the reaction (Table 4).

Fig. 3 summarizes and compares the behaviour of the three different antisera in the presence of IgG, Fc and Fab. Undegraded IgG is the only inhibitor which is equally effective in inhibiting each of the three different antisera used.

DISCUSSION

The antigens used for the inhibition of anti-globulin serum were pure by the criteria of immunoelectrophoresis, double immunodiffusion and ultracentrifugation. The anti-globulin sera used gave strong precipitin lines when reacted with IgG, Fab or Fc (Fig. 1a) while the anti-Fab gave no precipitin reaction with Fc (Fig. 2b).

The agglutinating system studied in these experiments involved red cells sensitized with incomplete anti-D. Under the same conditions, Rh negative cells were not agglutinated and neither were the Rh positive cells treated by IgG not containing anti-D antibodies. This anti-globulin test clearly involves the reaction between rabbit or sheep anti-human IgG antibodies and human IgG (anti-D), which sensitized the red cells. This is proved by the ease of inhibition of the reaction. The possibility that the agglutination involves one of the other immunoglobulins and is only inhibited by IgG due to a cross-reaction between IgG and the other immunoglobulins is ruled out by the inhibition of the reaction by Fc, that portion of the molecule which is unique to IgG (Table 2).

The minimal inhibition of the reaction produced by Fab is easily understood on the basis that this fragment of IgG can only react with a portion of the anti-IgG antibodies and therefore would not be expected to change the titre by more than one or two doubling dilutions. In addition, the fact that 170 μg of Fab does not inhibit more than does 2·7 μg of IgG and that 60 μg of Fab gives the same result as 1 μg Fc, shows that the Fab cannot contain more than 1–2 per cent impurity. Fc, which also reacts with only a portion of the anti-IgG antibodies, gives anomalous results. Fc and IgG are equally effective on a quantitative basis as inhibitors of the anti-IgG sera. On the other hand, these preparations of Fc do not inhibit appreciably the anti-Fab serum which is easily inhibited by Fab or IgG. The data indicate that contamination of Fc by IgG or Fab cannot be greater than 1 per cent by weight. It can therefore be assumed that in each of the four anti-globulin sera tested (Table 1), the antibodies, which are responsible for the agglutination of the anti-D sensitized cells, are those anti-IgG antibodies, which react with the antigenic determinants, common to IgG and Fc. The anti-globulin sera could, by chance, not contain appreciable quantities of antibodies reacting with the antigenic groups of Fab. That this is not the case in these experiments is clearly shown by the reaction in agar gel in which a strong precipitin line develops with Fab and by the fact that the sheep anti-IgG serum contains 2·2 mg/ml of antibody precipitable by Fab. Another possible explanation could be that, for steric or other reasons, the antigenic groups of the anti-D molecules, which correspond to those carried by Fab, are not available to react with their corresponding antibodies in the

anti-globulin serum or that this reaction does not lead to agglutination. That this is not the case for *all* the antigenic groups corresponding to Fab is shown by the experiments using anti-Fab antibodies (Table 3). Inhibition studies show that this antiserum produces an agglutination of the anti-D sensitized red cells through a specific reaction with the antigenic groups shared by the IgG anti-D and the fragment Fab. Such groups are therefore available and can react to produce an agglutination.

The difference in the behaviour of the anti-Fab antibodies as compared to the anti-IgG antibodies corresponding to the antigenic sites shared by Fab and IgG is difficult to explain. It does not seem to be merely a quantitative difference as each of the anti-globulin sera was rich in precipitating antibodies reacting with Fab. It does not seem possible that this reaction involves antibodies to antigenic groups which are exposed by the enzymatic degradation, because the reaction is completely inhibited by undegraded IgG (Table 3).

A more probable explanation might be based on differences in the specificities of the antibodies in the antiserum formed against Fab and those in the anti-IgG sera which react with Fab. It is known (Kunkel, Gray and Solomon 1966) that by the injection of IgG it is difficult to elicit the formation of antibodies which react with the Fd portion of Fab. The reasons for this are becoming clear: Fd is composed, at least in part, of that portion of the γ chains which seem to be highly variable in structure from one IgG molecule to another (Frangione and Franklin, 1965; Franklin and Frangione, 1966). It is also known that the N terminal half of the light chains have variable amino acid sequences (Putnam and Easley, 1965; Hilschmann and Craig, 1965) and presumably many different potential antigenic groups. The C terminal half can exist in either of two different structures corresponding to λ or κ chains. Thus, the Fab fragment of IgG is composed of a mixture of molecules having only a small part of their structure in common and therefore sharing only a few antigenic determinants. In the precipitin reaction these few antigenic determinants and the antibodies of corresponding specificity could precipitate and in so doing cause the co-precipitation of most of the other antibodies specific for the variable antigenic groups. For these antibodies, specific for the variable antigenic groups, to take part in an haemagglutination reaction, they must react with two anti-D molecules of the same structure on different red cells. Thus the bulk of the anti-Fab antibodies would not be expected to play a very important role in an agglutination reaction. The agglutination results obtained with the anti-Fab serum could be explained on the basis that this serum was rich in those antibodies which are specific for that portion of Fab which is common to all the IgG (anti-D) molecules on the sensitized red cells. That immunization by Fab tends to produce more antibodies reacting with the antigenic groups of Fab common to all IgG molecules has been noted previously (Kunkel *et al.*, 1966).

The mixing of the anti-IgG serum, reacting mainly with the antigenic groups shared by IgG and Fc, with the anti-Fab serum, which reacted with the antigenic groups shared by IgG and Fab, produced an anti-globulin serum reacting with both types of antigenic groups. It is not surprising, therefore, that this anti-serum could not be inhibited more than a few dilutions by either Fc or Fab as neither of these substances can react with all the anti-IgG antibodies causing the agglutination.

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