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EXPERIMENTALLY PRODUCED ANTIBODIES TO THE PEPSIN SITE OF IgG DUE TO UNTREATED AUTOLOGOUS IgG IN IMMUNE COMPLEXES

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SUMMARY

By immunizing rabbits with immune precipitates containing autologous IgG as the antibody part, most animals produced both rheumatoid factor-like antibodies and antibodies to human and rabbit $F(ab')_2$ IgG. The latter antibodies resembled the human antibodies to the pepsin site of IgG by not reacting with untreated IgG and by reacting with immune precipitates made from autologous and homologous antibodies. The rabbit antibodies to the pepsin site of IgG in most cases consisted of a mixture of IgG and IgM, while the human antibody is usually pure IgG.

The investigation demonstrated that in addition to rheumatoid factor, other human anti-IgG antibodies may also be due to antigenic stimulation by autologous IgG in immune complexes.

INTRODUCTION

Rheumatoid factor (RF) is usually considered as an antibody due to antigenic stimulation by autologous IgG. This concept is mainly based on studies on experimental production of RF-like antibodies. Such antibodies may be produced by immunizing rabbits with various forms of autologous denatured IgG (Milgrom & Witebsky, 1960; McCluskey, Miller & Benacerraf, 1962; Catsoulis *et al.*, 1965; Williams & Kunkel, 1965), IgG as antibodies in immune precipitates (Williams & Kunkel, 1963), or with heterologous antigens (Abruzzo & Christian, 1961; Aho & Wager, 1961; Christian, 1963).

Besides RF, human sera may contain other anti-IgG antibodies (Kunkel & Tan, 1964). Among these is the antibody to the so-called pepsin site, i.e. the structures revealed by pepsin treatment of IgG (Osterland, Harboe & Kunkel, 1963). The origin of this type of antibody is less firmly established than that of RF. However, it might also be due to immunization against autologous IgG, since rabbits immunized with bacteria (Christian, 1963) or various forms of autologous IgG (Williams & Kunkel, 1965) seemed to produce a similar antibody.

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The antibody part of immune complexes is the most likely source of altered IgG *in vivo*, and antibodies to the pepsin site of IgG occur in diseases involving prolonged immunization (Osterland *et al.*, 1963). Since such antibodies react with immune precipitates (Harboe, Rau & Aho, 1965), it is possible that they may originate from immunization with immune complexes.

The main purpose of the present investigation was to see whether antibodies to the pepsin site of IgG could be produced experimentally in rabbits by immunization with immune precipitates containing autologous antibodies. The animals were also tested for production of RF-like antibodies and for the specificity of the anti-IgG antibodies produced. For comparison, human antibodies to the pepsin site were tested for heterospecificity and ability to react with immune precipitates.

MATERIALS AND METHODS

Immunization procedure. Twelve white land rabbits, each weighing approximately 3 kg, were used. Eight rabbits were first given six weekly intramuscular injections of 0·5 mg of horse spleen ferritin (1 × crystallized, Mann Research Laboratories, Orangeburg, N.Y., U.S.A.). Four injections were given with 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.). After the last injection, the amount of antiferritin produced was calculated by making an antigen–antibody precipitation curve for each antiserum. The animals were then further given twenty weekly intraperitoneal injections of immune precipitates. Each injection consisted of immune precipitate from 5 mg of horse spleen ferritin and the rabbit's own anti-ferritin. To the first and third injection of this series 1 ml of Freund's complete adjuvant was added.

As a control four rabbits were given Freund's complete adjuvant only, administered by the same route and with the same intervals as for the experimental group. One of these rabbits died for unknown reasons before testing.

Blood was drawn prior to immunization, 9 days after each of the two series of injections, and 12 weeks after the last injection. Serum was separated and stored at −20°C if not tested immediately. Before use, the sera were inactivated for 30 min at 56°C. In the control group, blood was drawn prior to the first injection and corresponding to the end of the immunization of the experimental group.

Phosphate buffered saline (*PBS*) was produced by adding one volume of a 0·1 M phosphate buffer at pH 7·4 to nine volumes of saline.

Ferritin–anti-ferritin precipitates were produced by mixing equivalent amounts of the two components as determined by a precipitation curve made for each antiserum. The mixture was kept at 37°C for 2 hr and overnight at +4°C. The precipitates were washed three times in PBS at +4°C, and if not used immediately, they were kept frozen at −20°C.

Protein concentrations were measured by the Folin method, using a modification by Lowry *et al.* (1951).

Titration of anti-IgG antibodies was performed by mixing one drop of doubling dilutions of serum, or serum fractions, with one drop of PBS and one drop of a 1% suspension of human group O R_1 R_2 red cells sensitized with: either (a) Rabbit IgG, using $\frac{1}{3}$ basal agglutination tirte (BAT) (Tönder, 1962) of rabbit antiserum to human red cells (kindly provided by Mme Poudliachouk, Institute Pasteur, Paris); (b) Pepsin treated rabbit IgG, using $\frac{1}{3}$ BAT of the same antiserum to human red cells after pepsin treatment; (c) Human IgG, using human

anti-CD serum Ripley (kindly provided by Dr Marion Waller, Richmond, Virginia, U.S.A.), or (d) Pepsin treated human IgG, using the same anti-CD Ripley serum after pepsin treatment.

Testing of activities against red cells sensitized with rabbit IgG was performed on glass slides, which were kept at room temperature in a moist chamber for 10 min and then read macroscopically for agglutination. The activities against cells sensitized with human IgG were tested in tubes, which after 10 min at room temperature were centrifuged at 1000 *g* for 45 sec and read macroscopically for agglutination by gently tapping the tube. Agglutination strength was graded from 4+ to (+), and the titre recorded as the reciprocal of the highest original dilution giving a (+) reaction.

The lowest serum dilution tested was 1:4. Only sera which agglutinated unsensitized human red cells at this dilution were absorbed with such cells prior to testing for anti-IgG activity.

In inhibition experiments one drop of serum dilution was mixed with one drop of the solution to be tested for inhibition. After 5 min one drop of red cell suspension was added, and the procedure was continued as for the agglutination tests.

Absorption with immune precipitates containing autologous antiferritin was performed as described by Harboe *et al.* (1965), using approximately 3 mg of precipitate suspended in 0·2 ml of PBS for absorption of 0·2 ml of rabbit serum. The mixture was kept overnight at room temperature before removal of the precipitate by spinning.

Immune precipitates containing human anti-diphtheria toxoid were kindly provided by Drk. Aho, Helsingfors, Finnland, and kept at −20°C until used.

Density gradient ultracentrifugation was performed in a Spinco preparative ultracentrifuge type L50, using the technique of Fudenberg & Kunkel (1957). The sucrose concentrations ranged from 10 to 40%. Prior to testing, the fractions obtained were dialysed for 24 hr against PBS.

Splitting of disulphide bonds was performed with 2-mercaptoethanol (Sigma Chemical Company, St. Louis, Missouri, U.S.A.). Whole serum was diluted 1:2 in PBS and 2-mercapto-ethanol added to a final concentration of 0·2 M. The mixture was kept at room temperature for 2 hr and then tested for agglutinating activity. Untreated sera were tested simultaneously as controls.

Pepsin treatment of IgG and immune precipitates was performed as previously described (Natvig, 1966).

Aggregated IgG was produced by heating a 10 mg/ml solution of human Fr II IgG (obtained from Kabi A/S, Stockholm, Sweden) or rabbit Fr II IgG (kindly provided by Dr O. Tönder, Bergen, Norway) for 10 min at 63°C.

Coating of human red cells with human serum albumin (obtained from Behringwerke, Marburg, Lahn, West Germany) was performed by using bis-diazotized benzidine as described by Natvig & Kunkel (1968).

Testing for antigenic markers of the IgG molecule was performed with the same technique and antisera as described by Natvig, Kunkel & Joslin (1969).

RESULTS

Anti-IgG antibodies resulting from immunization with immune precipitates

Eight rabbits were tested for their content of antibodies to human red cells sensitized with: either (a) rabbit IgG, (b) pepsin treated rabbit IgG (rabbit $F(ab')_2$ IgG), (c) human IgG or

(d) pepsin treated human IgG (human $F(ab')_2$ IgG). The tests were performed before immunization and after immunization with immune precipitates containing autologous anti-ferritin. Testing after the initial immunization with horse spleen ferritin alone was usually omitted, since pilot experiments showed that there was no significant difference between the results in sera obtained at this stage and those in pre-immune sera. The immunization scheme and a typical result are shown in Fig. 1, while Fig. 2 summarizes the results

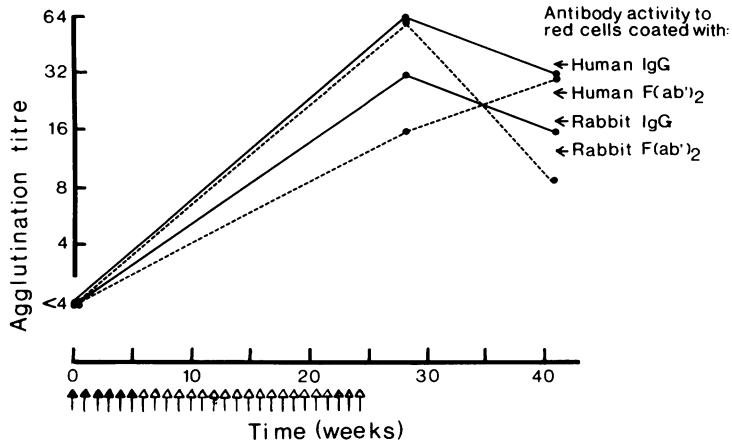

FIG. 1. Anti-IgG activity in serum obtained before and after immunization of a rabbit (K 332) with horse spleen ferritin (closed arrows) and immune precipitates made from autologous anti-horse spleen ferritin (open arrows).

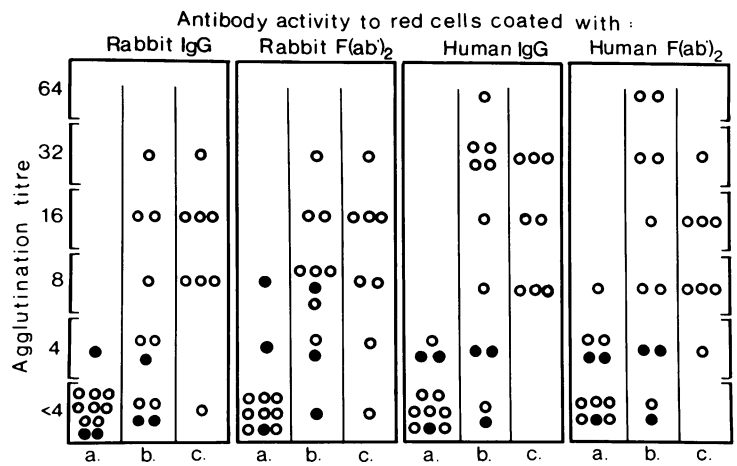

FIG. 2. Anti-IgG activity in rabbits immunized with immune precipitates made from autologous anti-horse spleen ferritin (open circles) or with Freund's complete adjuvant (closed circles). Sera were tested before immunization (a), at the end of immunization (b), and 12 weeks later (c).

in all animals. Prior to the immunization there was usually no antibody activity against the coated cells. In three rabbits there was a very weak activity against human $F(ab')_2$ IgG.

After immunization the titre of one or more of the anti-IgG antibodies was increased in all animals by two or more titration steps. Since similar results were obtained in repeated tests, an increase by two titration steps was judged to be significant. The titre against human IgG increased somewhat more frequently and strongly than that against rabbit IgG. The increase in antibody activity against the types of $F(ab')_2$ IgG was very similar to that against IgG, even in the individual rabbit.

Twelve weeks later the titres in most cases were unchanged or had decreased slightly. In a few cases the titre had increased by one titration step. The experiment therefore demonstrated that both antibodies to human and rabbit IgG and antibodies reacting with the corresponding pepsin treated proteins were produced after immunization with immune precipitates containing autologous antibody. In the control animals immunized with Freund's adjuvant only, there was usually no anti-IgG activity, either before or after immunization. In a few cases the titre had increased or decreased by one titration step, which was judged to be insignificant.

All the immune sera were further tested for the ability to precipitate aggregated human and rabbit IgG. In double diffusion experiments in agar, no precipitation occurred. However, most of the sera produced a small amount of precipitate when making a precipitation curve with aggregated human IgG. There was no significant precipitation with aggregated rabbit IgG.

Inhibition experiments

To test the specificity of the anti-IgG antibodies obtained, the five immune sera with the highest antibody activity were tested in inhibition experiments with the rabbit and human IgG, either whole or pepsin treated. Corresponding results were obtained in all five sera, and a typical example is shown in Table 1.

TABLE 1. Inhibition of anti-IgG activity in serum obtained from a rabbit (K 332) immunized with immune precipitates made from autologous anti-horse spleen ferritin

Solution tested for inhibiting activity	Agglutination titre against red cells coated with:			
	Rabbit IgG	Rabbit $F(ab')_2$ IgG	Human IgG	Human $F(ab')_2$ IgG
Rabbit IgG 1·0 mg/ml	16	16	32	32
Rabbit IgG 10·0 mg/ml	4	16	32	32
Rabbit $F(ab')_2$ IgG 0·6 mg/ml	16	<4	32	8
Human IgG 1·0 mg/ml	16	16	<4	32
Human $F(ab')_2$ IgG 0·6 mg/ml	16	4	32	4
Control: Saline	8	16	16	32

The antibody activities were always inhibited by the protein used for red cell coating in the respective test. The RF-like antibodies to red cells coated with rabbit and human IgG

were only inhibited by the homologous proteins, and a high concentration (10 mg/ml) of rabbit IgG was needed to inhibit the antibody to this protein. Even then only a partial inhibition was obtained. In contrast, the antibodies to red cells coated with rabbit and human $F(ab')_2$ IgG were easily inhibited by the homologous proteins and also partly inhibited by the heterologous pepsin treated IgG.

The inhibition experiments indicated that the rabbits produced specific antibodies to all the four types of IgG tested, but there was some cross-reaction between the heterospecific and isospecific antibody to pepsin treated IgG. The latter type of antibody probably reacted with the pepsin site of IgG, since it was not inhibited by the native molecule.

Absorption with immune precipitates

If the antibodies obtained during immunization were due to the precipitates used for immunization, one would expect that they could be removed by the same precipitates. Immune sera from four rabbits were therefore tested after absorption with ferritin–anti-ferritin precipitate containing antibody from the same rabbits. The results with one of the

TABLE 2. Absorption of anti-IgG activity in serum obtained from a rabbit (K 332) immunized with immune precipitates made from autologous anti-horse spleen ferritin

	Agglutination titre against red cells coated with:			
	Rabbit IgG	Rabbit $F(ab')_2$ IgG	Human IgG	Human $F(ab')_2$ IgG
Absorbed with immune precipitates made from autologous anti-ferritin complexes	<4	4	16	32
Unabsorbed	32	32	16	32

sera is shown in Table 2. In all cases the activity against pepsin treated and untreated rabbit IgG was absorbed, while there was only partial or no effect on the activity against the corresponding human proteins.

Determination of immunoglobulin class of anti-IgG antibodies

All the rabbit sera were tested for the four antibody activities before and after treatment with 2-mercapto-ethanol, which is known to destroy the agglutinating effect of IgM antibodies but not that of IgG antibodies (Fudenberg & Kunkel, 1957; Grubb & Swahn, 1958). After treatment there was always a reduction of the agglutinating activity, which in some cases was completely abolished.

Since the reduction with 2-mercapto-ethanol might also affect the $F(ab')_2$ IgG on the red cells, a further determination of the immunoglobulin class was performed by separating the immune sera from five of the rabbits with the highest antibody activity by density gradient ultracentrifugation. The fractions obtained were tested for the four anti-IgG activities. An example is shown in Fig. 3. The various activities were found corresponding either to the small bottom protein peak known to contain IgM, or to both this peak and the deepest part

of the large peak, known to contain IgG. There was no significant difference between the various antibodies.

These experiments therefore indicate that all the four anti-IgG activities tested were due either to a mixture of IgM and IgG antibodies or pure IgM antibodies.

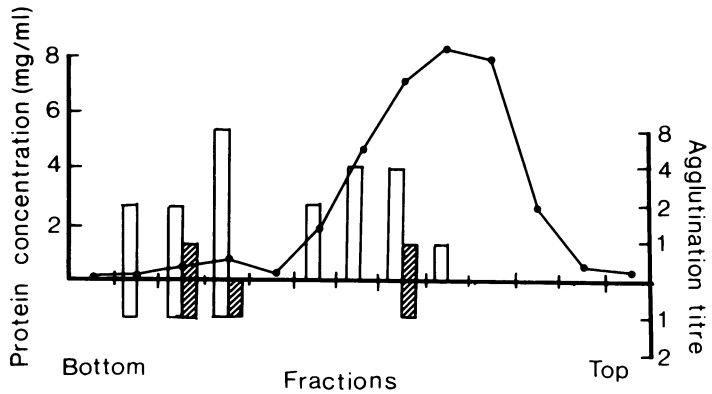

FIG. 3. Anti-IgG activity in fractions produced by density gradient centrifugation of serum obtained after immunization of a rabbit (K 334) with immune precipitates made from autologous anti-horse spleen ferritin.

The solid line shows the protein concentration, and the columns express the anti-IgG activity to: rabbit IgG (open columns above abscissa), rabbit $F(ab')_2$ IgG (hatched columns above abscissa), human IgG (open columns below abscissa), and human $F(ab')_2$ IgG (hatched columns below abscissa).

Purity of preparation used for immunization

Although care was taken not to contaminate the preparations used for immunization, the activity observed against human IgG might be due to immunization with human serum proteins. Such a contamination would most likely be due to human whole serum, which should stimulate the formation of antibodies also to other serum proteins. The rabbit sera were therefore screened for antibodies to human serum albumin by a passive haemagglutination technique which should have a sensitivity similar to that used for detecting anti-IgG antibodies. In no case was any anti-albumin activity observed, indicating that no significant contamination had taken place. It was also possible that the rabbit anti-IgG activities were due to contaminating horse immunoglobulin in the preparation of horse spleen ferritin used for immunization. However, this seemed improbable, as control experiments demonstrated that the ferritin did not inhibit the various rabbit anti-IgG antibodies.

Specificity of the reaction between immune precipitate and human antibody to the pepsin site of IgG

The reaction between rabbit antibodies to the pepsin site of IgG and immune precipitates resembled that previously demonstrated for the corresponding human antibodies (Harboe *et al.*, 1965). The latter observation was confirmed in experiments with eleven human sera containing antibodies to the pepsin site, which were all neutralized after inhibition with immune precipitates. This raised the question of whether the effect was due to the precipitates themselves or to $F(ab')_2$ fragments formed during their preparation. To test this possibility

immune precipitates made from human anti-diphtheria toxoid were tested in haemagglutination inhibition reactions for their Fd and Fc antigens. The tests were run before and after pepsin digestion of the precipitates and the endpoints of inhibition compared for the various test systems.

The results are shown in Table 3. In the intact precipitate the Fc antigen 'non a' and sometimes also Gm(a) were present together with the Fd antigen Gm(f). There was also a clear inhibition of the system for testing of the IgG1 pepsin site (Natvig, 1970). Following pepsin digestion this activity and that of Gm(f) were basically unchanged. In contrast, the activities of the Fc antigens 'non a', 'non g', Gm(b), Gm(g), and Gm(a) were abolished. The same strong inhibition of the antibodies to the pepsin site was preserved. In addition to a retention of Fd antigens and a major change, with complete loss of the Fc antigenic activity, this strongly suggests that the inhibition was due to the intact precipitates and not to minor

TABLE 3. Inhibition of human antibodies to the pepsin site and anti-Gm antibodies by immune precipitates

	Antibodies to the pepsin site of:		Anti-Gm antibodies specific for:					
	γG1	γG3	Gm (f)	Gm (a)	Gm (b)	Gm (g)	'non a'	'non g'
Precipitates:								
1.S Native	0·015	0·06	0·015	0·03	0·25	> 1·0	0·015	0·12
Pepsin digested	0·015	0·06	0·015	1·0	> 1·0	> 1·0	1·0	> 1·0
2.L Native	0·03	1·0	0·06	1·0	> 1·0	> 1·0	0·12	> 1·0
Pepsin digested	0·015	0·25	0·06	> 1·0	> 1·0	> 1·0	> 1·0	> 1·0
3.P Native	0·06	0·25	0·12	1·0	> 1·0	> 1·0	0·12	1·0
Pepsin digested	0·06	0·25	0·06	> 1·0	> 1·0	> 1·0	> 1·0	> 1·0
Control (pooled IgG):								
Native	> 1·0	> 1·0	0·03	0·016	0·12	0·12	0·03	0·06
Pepsin digested	0·015	0·03	0·015	> 1·0	> 1·0	> 1·0	> 1·0	> 1·0

The antigens detected by antibodies to the pepsin site are specific for the $F(ab')_2$ fragments of IgG1 and IgG3, respectively (Natvig, 1970).

Gm(f) resides in the Fab fragment of IgG1 proteins, Gm(a) in Fc of IgG1, Gm(b) and (g) in Fc of IgG3 (Natvig & Kunkel, 1968).

The 'non a' and 'non g' antigens are located in the Fc part of IgG1, 2, and 3 and IgG2 and 3, respectively (Natvig *et al.*, 1969).

The test systems used are those described in the papers mentioned above.

contamination of $F(ab')_2$ material in the precipitates. In the latter case one would expect a strong increase in inhibiting capacity paralleling the loss of Fc antigens.

Reaction between human sera and rabbit $F(ab')_2$ IgG

Since the present experiments indicated that the experimentally produced antibodies to the pepsin site of IgG reacted both with the rabbit and human protein, the corresponding antibodies in human sera were tested for reaction with heterologous $F(ab')_2$.

Only one out of the seventeen sera tested showed a clear agglutination reaction with red cells coated by rabbit $F(ab')_2$. This agglutination was inhibited by both rabbit and human $F(ab')_2$, indicating that it represented a cross-reacting antibody.

In contrast, the reaction of all the human antibodies against red cells coated by human $F(ab')_2$ IgG was inhibited by this protein and not by the corresponding rabbit protein.

DISCUSSION

By immunizing rabbits with immune precipitates containing autologous immunoglobulin as the antibody part, most animals produced anti-IgG antibodies reacting with red cells coated with rabbit and human $F(ab')_2$ IgG and other antibodies reacting with the corresponding native molecules. The investigation therefore demonstrated that in addition to RF-like antibodies, another type resembling the human antibody to the pepsin site of IgG may also be produced experimentally by immunization with immune precipitates.

The experimentally produced antibody to the pepsin site of IgG resembled the corresponding human antibody by reacting only with this site and not with the native molecule. The rabbit antibody reacted with approximately identical strength with $F(ab')_2$ from rabbit and human IgG, thus differing somewhat from the human antibody which reacted almost exclusively with the human IgG fragments. The inhibition experiments indicated that the reactions caused by the rabbit sera were due to two cross-reacting antibody populations; one reacting preferentially with the pepsin site of rabbit IgG and one with the corresponding site on human IgG. The rabbit antibody to the pepsin site of IgG also differed from the corresponding human antibody by being either IgM or a mixture of IgM and IgG, while the human antibody is nearly always IgG (Osterland *et al.*, 1963; Natvig, 1970).

The RF-like antibody observed in this and previous investigations with immune precipitates (William & Kunkel, 1963) seemed to be directed both against rabbit and human IgG. In experiments where autologous denatured IgG was used for immunization, the specificity was directed almost exclusively against human IgG (Milgrom & Witebski, 1960; McCluskey *et al.*, 1962), while when using pure heterologous protein other than IgG, the antibody reacted most strongly or exclusively with rabbit IgG. This indicates that the different specificity of rabbit and human antibody to pepsin split IgG may be due to small differences in the autologous IgG causing the antibody production.

The antibody titres observed in the present experiments were relatively low when compared with the titres of the RF-like antibodies obtained in previous experiments. This discrepancy may be due to our use of immune precipitates produced at antigen–antibody equivalence. The alteration in the IgG molecules in such precipitates may not be optimal for production of anti-IgG antibodies. When heterologous antigens are used for immunization, probably a whole spectrum of antigen–antibody complexes with different molecular ratios of antigen to antibody will be produced, some of which may be more auto-antigenic than the precipitates. Another factor may be the rather weak sensitization of the red cells used in the haemagglutination tests, causing a relatively insensitive test system. A high concentration of rabbit IgG was needed to inhibit the activity against cells coated with this protein. Although it may also be difficult to inhibit human RF with human IgG, the observed preference for antigen bound antibody suggests that this anti-IgG in the rabbit is more similar to a Milgrom type of anti-antibody (Milgrom, Dubiski & Woznicko, 1956) which is known to react only with antibody fixed to its antigen.

The absorption experiments with autologous immune precipitates demonstrated that the antibodies produced to pepsin treated and untreated rabbit IgG reacted with the precipitates used for immunization, while it was difficult to absorb out the activity to the corresponding human proteins. The reason for the latter observation is not clear, especially as it was easy to inhibit the activity against cells coated with human IgG. The failure to absorb the activity against human $F(ab')_2$ IgG might be explained if the immune precipitates do not themselves contain the relevant antigen, this being revealed during the destruction *in vivo* of the precipitates. The rabbit antibody to the pepsin site of rabbit IgG also resembled the corresponding human antibody in that it could be removed by absorption with a precipitate containing autologous or homologous IgG as the antibody part. The antibody to the pepsin site of IgG probably reacts with the precipitate itself and not with fragments formed during the preparations. This was indicated by the inhibition experiments with pepsin treated immune precipitate, as there was no increase of inhibition after pepsin treatment.

All normal rabbits contain an antibody reacting with autologous papain treated IgG (Mandy, 1966). This so-called 'homoreactant' is probably different from the antibody to the pepsin site of IgG observed in the present experiments, since only trace amounts of the latter antibody were present in a few rabbits prior to immunization.

The investigation showed that like RF the human antibody to the pepsin site of IgG may be due to antigenic stimulation by autologous IgG altered by fixation to its antigen. This would mean that a structure which is 'buried' in the intact IgG molecule may be revealed both by pepsin treatment and fixation to the specific antigen in immune precipitates.

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