PROPERTIES OF VARIOUS ANTI-γ-GLOBULIN FACTORS IN HUMAN SERA*,‡

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A wide variety of anti-γ-globulin factors have been demonstrated in human sera. The serological and physicochemical properties of most of these factors are known in considerable detail, whereas others have been less well characterized.

The so called rheumatoid factors consist of 19S γM-globulins which in whole serum form a 22S complex through interaction with γG-globulin (1, 2). Various test systems demonstrate an array of different factors that partly can be separated by absorption and elution procedures (3–5). These factors react with the Fc fragment of rabbit and/or human γG-globulin (6–8).

Rheumatoid factors in some human sera demonstrate genetic factors on human γG-globulin (9, 10). These "anti-Gm factors" react with the Fc fragment of γG-globulin (11–13). The serological properties of anti-Gm factors in rheumatoid and non-rheumatoid sera are distinct (14, 15); in the latter case, they may be typical isoantibodies resulting from stimulation by foreign type γG-globulin introduced by blood transfusions (16) or transplacental passage (17–19).

It is not clear whether "anti-Inv factors" should be called rheumatoid factors. They are, at least in the majority of cases, found in non-rheumatoid sera and react with determinants present in the Fab fragment of γG-globulin (11, 12). Other anti-γ-globulin factors reacting with determinants present on the light chains of γG-globulin have recently been described by Williams (20). Tanned cells coated with isolated light chains of human γG-globulin are agglutinated by some rheumatoid sera. This agglutination is inhibited by isolated light chain preparations, but not by intact γG-globulin, indicating that the factors react with determinants that are hidden in the intact molecule.

Milgrom *et al.* (21) observed that some human sera agglutinated red cells coated

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with incomplete anti-Rh antibodies, and that the agglutination was not inhibited by addition of pooled human γG-globulin. The latter phenomenon showed that the active substance was serologically distinct from rheumatoid factors. Sera with identical serological properties were described independently by Grubb (9) under the term "non-rheumatoid type agglutinators". The term "anti-antibody" was used by Milgrom *et al.* (21). More recent data indicate, however, that a wide variety of substances are able to interact with antibody γ-globulin in such a way that they logically might be referred to as anti-antibodies. It is felt, therefore, that the term "anti-antibody" should not be restricted to only one of these substances. Until more complete characterization of the various substances is achieved, it is suggested that a more noncommital designation be used. To denote the factor originally referred to as "anti-antibody" by Milgrom *et al.* (21), we shall use the term "Milgrom type anti-γ-globulin".

Finally, Osterland *et al.* (8) observed that some human sera were able to agglutinate red cells sensitized with pepsin-digested incomplete anti-Rh antibodies, whereas they did not agglutinate cells sensitized with intact anti-Rh antibody. The responsible factor could be separated from the other anti-γ-globulin factors by several techniques. It did not react with native γG-globulin but apparently with a hidden determinant that was exposed by digestion with pepsin or papain at low pH.

The present investigations were undertaken to compare the properties of various anti-γ-globulin factors in human sera. Rheumatoid factors, Milgrom type anti-γ-globulin factors, and antibodies directed against an enzyme revealed antigen in human γG-globulin were studied. It was demonstrated that the hidden antigen was exposed, not only by the somewhat artificial procedure of enzymatic digestion at low pH, but also when human γG-globulin functioned as antibody in an antigen-antibody precipitate. The results obtained indicate that the three different forms of anti-γ-globulin factors may all be produced as a result of antigenic stimulation by autologous antigen-antibody complexes.

Materials and Methods

Human Sera.—"Normal sera" were obtained from healthy blood donors. About 750 sera from a "mixed hospital population" were tested. These patients had been admitted to the surgical and medical departments of Oslo City Hospital for a variety of disorders, and sera were obtained in connection with blood typing or compatibility tests prior to blood transfusion. They were screened for the presence of Milgrom type anti-γ-globulin factors using red cells coated with three different anti-Rh antibodies selected for reasons given below. Five potent sera (Be., En., He., Ni., Sv.) were found and used in the present investigations. Rheumatoid sera were obtained through the courtesy of Dr. V. Laine of The Rheumatism Foundation Hospital, Heinola, Finland. They were from patients with rheumatoid arthritis having peripheral, symmetrical joint lesions, and positive Waaler-Rose and latex fixation reactions. The sera were selected on the basis of these criteria since patients with similar joint lesions and negative serological reactions often appear to suffer from other diseases than rheumatoid arthritis (22). The sera were stored in small aliquots at −25°C until used.

Tests for Rheumatoid Factors.—The Waaler-Rose and latex fixation tests were performed by techniques described elsewhere (15, 23). Agglutination experiments with red cells coated with

incomplete anti-Rh antibodies were performed on slides. Three drops were introduced in each reaction; one drop of the serum dilution, one drop of saline, and one drop of coated red cells. The titres are given as reciprocals of the dilutions of sera when introduced into the mixture. In *inhibition tests*, the drop of saline was replaced by one drop of the material to be tested for inhibiting capacity. In most experiments, the extra drop of γ-globulin in saline at a concentration of 1 mg/ml was added to a series of dilutions of serum. The agglutination titre was recorded and compared with that of the control sample where only saline was added. In some instances, a fixed dilution of serum containing 8 to 10 agglutinating doses was tested with serial dilutions of the material to be examined for inhibiting capacity. Controls were always included as in inhibition experiments for determination of Gm types (24).

Coating of Red Cells with Various Antibody Preparations.—Coating of red cells with incomplete anti-Rh antibodies was performed as previously described (27). Red cells coated with anti-CD Ripley were used for the detection of rheumatoid factor since this reagent is known to react with most rheumatoid factors. For reaction with Milgrom type anti-γ-globulin factors, cells coated with incomplete anti-Rh antibodies Ho., Hu., Lo., and Ni. were used. These antisera were selected since cells coated in this way had previously been shown to react with only a minority of rheumatoid factors and at the same time to react well with Milgrom type anti-γ-globulin factors.

To obtain anti-A and anti-B antibodies of γG- and γM-globulin type from the same serum, an individual of group O was immunized with blood group AB substance (Merck Sharp and Dohme, Amsterdam, The Netherlands) as described elsewhere (25). One part of the serum was fractionated by DEAE cellulose chromatography and a pure γG-globulin fraction was obtained. Another part of the serum was subjected to gel filtration on a sephadex G-200 column to obtain the macroglobulin fraction (26). Group A_1B red cells were subsequently sensitized with various subagglutinating doses ($\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$ of the minimum agglutinating dose) of the two kinds of isoagglutinins. Agglutination tests with these cells and selected sera were performed on slides using pooled normal, rheumatoid factor–free serum as control.

Coating of red cells with pepsin-digested anti-CD Ripley was performed as described elsewhere (8), and only some details on the present preparation are given. The γ-globulin fraction of serum Ripley was obtained by precipitation with $(NH_4)_2SO_4$ and subsequently digested with pepsin (pepsin, 2 times crystallized, Worthington Biochemical Corporation, Freehold, New Jersey) at pH 4.1. After digestion, precipitin tests in gel with specific anti-Fab and anti-Fc antisera indicated that the Fc part of the γG-globulin molecule had been completely destroyed whereas the Fab part was intact. The reactions of cells coated with whole anti-CD Ripley were then compared with those of cells coated with the pepsin-digested anti-CD Ripley: agglutination tests using serial dilutions of the anti-Fab serum and varying amounts of antibody to coat the cells, showed that the agglutination was equally strong of cells coated with pepsin-digested anti-CD Ripley as of cells coated with γ-globulin isolated from serum Ripley and exposed to low pH only. In marked contrast, anti-Fc did not agglutinate red cells coated with the pepsin-digested antibody, whereas cells coated with whole anti-CD Ripley were strongly agglutinated. To get optimal conditions for comparative analyses, only one preparation of pepsin-digested anti-CD Ripley was used for the experiments to be described in this paper. After the digestion and the various controls had been completed, the material was divided in small aliquots and stored at −25°C. Each sample was thawed only once for coating of red cells. In parallel with the digestion of isolated γ-globulin from serum Ripley, a sample of pooled human γG-globulin (AB Kabi, Stockholm, Sweden) was digested under identical conditions. After digestion, this preparation could no longer inhibit a reference anti-Gm(a) serum which is an additional indication that the digestion had been complete (8). The preparation was stored in small aliquots in the frozen state and used for inhibition tests.

Human γG-globulin was obtained commercially from AB Kabi. Soluble γ-globulin aggregates were prepared by heating or by treatment with mercaptoethanol and urea as described by Edelman *et al.* (27).

Reduction by Mercaptoethanol.—Serum was diluted 1:2 in saline. 2-Mercaptoethanol (Fluka AG, Buchs, Switzerland) was added to a final concentration of 0.15 M followed by incubation overnight at room temperature.

DEAE Cellulose Chromatography was performed using cellulose *N*,*N*-diethylaminoethyl ether (Eastman Organic Chemicals, Rochester, New York) and a stepwise elution technique with phosphate buffers of pH 6.8 of varying molarity. The fractions representing each peak of eluted protein were pooled and tested for the various serological activities after concentration by ultrafiltration and dialysis against saline. Tests for purity were performed by immunoelectrophoresis using an anti-whole human serum (Behringwerke AG., Marburg-Lahn, Germany) and by diffusion in gel techniques with antisera specific for each of the immunoglobulins.

Density Gradient Ultracentrifugation was carried out by the technique described by Kunkel *et al.* (28).

Antisera.—Rabbit antisera were obtained by immunization with purified protein preparations in complete Freund's adjuvant. By various absorption procedures, the antisera were made specific for γG-, γA- and γM-globulin respectively. This was verified by diffusion in gel experiments with whole human serum and the respective purified proteins. Anti-Fab was obtained by immunization with pepsin-digested γG-globulin (8). The resulting antiserum gave a single line with whole human serum by immunoelectrophoresis and did only precipitate with the Fab fragment after papain digestion of human γG-globulin. Anti-Fc was obtained by absorption of an anti-γG-globulin with pepsin-digested γG-globulin. Its specificity was verified by immunoelectrophoretic analyses with intact and papain-digested γG-globulin. Human diphtheria antitoxin was obtained by immunization of volunteers as previously described (29). Eight different antisera were used and these gave identical results in the absorption experiments to be described.

Absorption Experiments.—For absorption experiments with cells sensitized with γG-globulin antibodies, cells were coated as described above. Aliquots of a serum containing a Milgrom type anti-γ-globulin factor were diluted 1:2 and mixed with equal volumes of cells sensitized with the anti-Rh antibodies and with the anti-AB antibodies (the latter corresponding to ½ of minimum agglutinating dose). After 2 hours' incubation at room temperature the supernatants were obtained by centrifugation and tested for serological activity in parallel with controls that had been exposed to non-sensitized cells.

For absorption experiments with cells sensitized with γM-globulin antibodies, human anti-Salmonella O was used. These antibodies are known to belong to the 19S class in most cases (30). One convalescent serum with an anti-O titer of 500 was used. It was verified that its agglutinating ability was completely abolished by reduction with mercaptoethanol. Three ml of Salmonella group B (1, 4, 5, 12) O suspension, corresponding to 60 ml of the OB antigen suspension used in the Widal reaction for Salmonella agglutinins (31) and 3 ml of convalescent serum were incubated for 2 hours at 37°C, followed by washing three times in saline. The packed, sensitized bacteria were used for absorption of 0.2 ml of each of two sera for 2 hours at room temperature. The supernatants were collected by centrifugation and tested for agglutinating ability in parallel with controls that had been exposed to non-sensitized bacteria.

For absorption experiments with antigen-antibody precipitates containing rabbit antibody, quantitative precipitin curves were set up to define the conditions of maximal precipitation. Antigen and the corresponding antiserum were then mixed in these proportions. The precipitates were collected by centrifugation and washed three times in saline to be used for absorption. Techniques for preparation of precipitates consisting of diphtheria toxoid and human antitoxin are described elsewhere (29). Three mg of washed, packed precipitate were incubated

with 0.1 ml of serum and 0.1 ml of saline overnight at room temperature. Care was taken to obtain an even suspension of precipitate in the serum to get optimal conditions for absorption. Controls consisted of sera incubated with saline alone under identical conditions.

EXPERIMENTS AND RESULTS

Properties of Milgrom Type Anti-γ-Globulin Factors.—Five sera were found that agglutinated red cells sensitized with any of seven strong incomplete anti-Rh antibodies. Incubation of unsensitized cells and incomplete anti-Rh serum with these sera resulted in agglutination identical with that obtained by using washed, previously sensitized cells. The agglutination was not inhibited by addition of pooled human γG-globulin. These properties are identical with those of the sera originally described by Milgrom *et al.* (21).

In all cases, reduction with mercaptoethanol resulted in complete loss of agglutinating ability, indicating that the active substance was a γM-globulin (28). Confirmation was sought by exposing the sera to density gradient ultracentrifugation and DEAE cellulose chromatography. In each case, activity was recovered only from the fractions that contained γM-globulin.

A series of experiments were designed to see whether the active substance reacted with the Fab or the Fc part of γG-globulin.

Inhibition experiments were made using γG-globulin that had been aggregated in various ways. As described, pooled human γG-globulin did not inhibit the agglutination. γG-Globulin aggregated by heating or by treatment with mercaptoethanol and urea also had only minimal inhibiting capacity. By adding these materials in a concentration of 1 mg/ml to each of serial twofold dilutions of sera containing potent Milgrom type anti-γ-globulin factors, the agglutination titre remained unchanged or was reduced only one step. As a control, rheumatoid sera were tested similarly using cells coated with anti-CD Ripley as indicator. In this instance, the aggregated γG-globulin preparations reduced the titre 7 to 8 steps. Attempts were made to aggregate isolated Fab fragments, but no significant inhibiting ability was obtained.

γG- and γM-globulins cross-react because of common antigenic determinants which are located on the Fab part of γG-globulin (32, 33). Agglutination and absorption experiments with red cells sensitized with antibodies belonging to different immunoglobulin classes might therefore give valuable information. The ability of an anti-γ-globulin factor to interact with cells sensitized with both γG- and γM-globulin antibodies would indicate reactivity with the Fab part, whereas reaction only with cells coated with γG-globulin antibodies would indicate reactivity with the Fc part of γG-globulin which is specific for this protein.

Group O individuals were immunized with blood group substance and a serum with high titre anti-A and anti-B antibodies was subjected to gel filtration on sephadex G-200 and DEAE cellulose chromatography. Cells sensi-

tized with γG-type anti-AB were agglutinated by Milgrom type anti–γ-globulin factors, whereas no reactivity was observed with the cells coated with γM-globulin anti-AB.

Similar absorption experiments were then performed. Red cells sensitized with γG-globulin antibodies (incomplete anti-Rh antibodies and anti-AB antibodies isolated by chromatography) did absorb Milgrom type anti–γ-globulin factors.[1] For evaluation of γM-globulin antibodies, Salmonella group B bacteria sensitized with γM-globulin type anti-O antibodies were used. Absorption of the same sera with this material did not reduce the titre.

Pepsin digestion of human γG-globulin and similar type specific antibodies results in a fragment with antibody activity that structurally is very similar to the isolated Fab fragment after papain digestion (8). Agglutination experiments using Rh positive red cells coated with incomplete anti-Rh antibodies, or such antibodies digested with pepsin, thus provide a useful means for comparison of interactions between various agglutinating factors and the whole γ-globulin molecule or the isolated Fab fragment. The reactivity of sera with Milgrom type anti–γ-globulin factors and cells coated with anti-CD Ripley or pepsin-digested anti-CD Ripley was therefore tested. The five selected sera agglutinated red cells coated with whole anti-CD Ripley. The agglutination was not inhibited by γG-globulin or by pepsin-digested γG-globulin. The sera did also agglutinate red cells coated with pepsin-digested anti-CD Ripley. Inhibition experiments were made to characterize the substance responsible for the latter type of agglutination. It was observed that this agglutination was not inhibited by native γG-globulin, whereas pepsin-digested γG-globulin had a marked inhibiting capacity. These findings indicated that each serum contained two distinct anti–γ-globulin factors.

To obtain a fraction that only contained the Milgrom type anti–γ-globulin factor, attempts were made to separate the two types of activity from individual sera. The behavior of such a fraction against the two types of coated cells might then give valuable information.

Fig. 1 illustrates the findings on serum En. where a complete separation was obtained. Activity against cells coated with pepsin-digested anti-CD Ripley was recovered in the first 3 peaks. The first peak contained only γG-globulin. The activity in the second peak was attributed to the content of γG-globulin in this fraction. The third peak contained the bulk of the serum's γA-globulin and had a marked agglutinating activity against cells coated with pepsin-

[1] Absorption with the relatively small amount of cells sensitized with incomplete anti-Rh antibodies abolished the agglutinating activity against cells sensitized with the anti-Rh antibodies, leaving the agglutinating activity against cells sensitized with the anti-AB antibodies essentially unchanged and *vice versa*. Thus it seems that the Milgrom type anti–γ-globulin factor in a single serum may consist of a heterogeneous population of molecules that partly can be separated by absorption procedures.

digested anti-CD Ripley. The titre was higher than that of the first peak. Experiments using antisera that react specifically with γG-globulin indicated that the third peak contained about 1/100 of the amount of γG-globulin present in the first peak. Considering the titers, it was therefore judged that the agglutinating ability resided in the γA-globulin. Agglutinating activity against cells coated with whole anti-CD Ripley was confined to the fourth peak

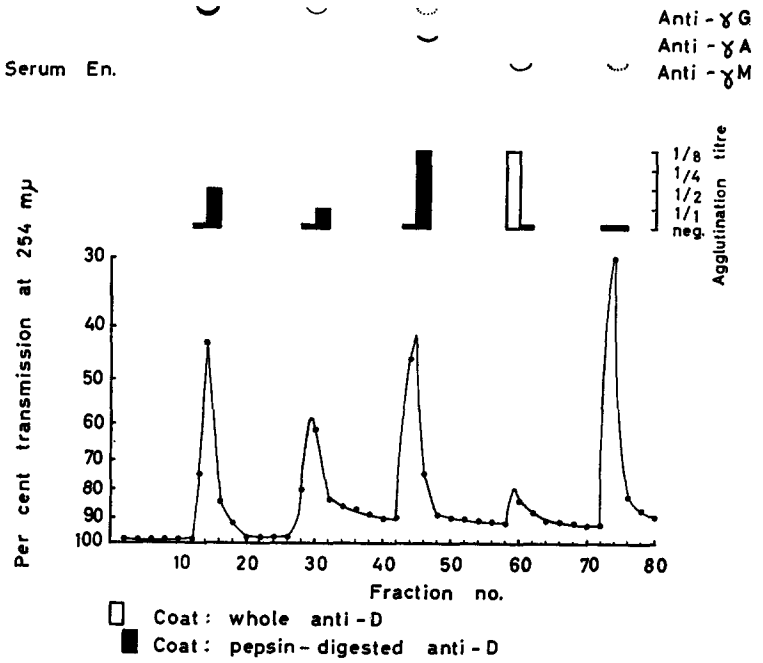

FIG. 1. Fractionation of serum En. by DEAE cellulose chromatography. The various fractions were tested for protein content by precipitin tests with antisera reacting specifically with the three main immunoglobulins and for serological activity by various agglutination tests as indicated.

which contained the bulk of the serum's γM-globulin. Repeated experiments confirmed the observation that the agglutinating factor recovered with this peak reacted with red cells coated with whole anti-CD Ripley, whereas cells coated with pepsin-digested anti-CD Ripley were not agglutinated. This finding again indicated that the Milgrom type anti-γ-globulin factor reacted with the Fc fragment of γG-globulin.

Properties of Agglutinator Active Against Cells Coated with Pepsin Digested Anti-CD Ripley.—Since only limited information is available (9), a survey was made for this agglutinator in various types of sera. To save reagents, the sera were tested only in dilution 1:5 and the reactions were read after 10 minutes'

incubation. This was considered feasible since no prozone phenomenon has been observed so far with this type of agglutinator. The results are summarized in Table I. It appears that the frequency of this agglutinator was fairly low in sera of blood donors, whilst it occurred about twice as often in sera from a mixed hospital population. Sera from patients with rheumatoid arthritis often contained the factor. A striking observation was that all of the five sera with a Milgrom type anti-γ-globulin factor also contained potent agglutinators active against cells coated with pepsin-digested anti-Rh antibody.

Inhibition experiments confirmed the previous observations (8) that the agglutination is not inhibited by intact γG-globulin but only by γG-globulin digested with pepsin or papain at low pH. This behavior sharply distinguishes this agglutinator from other anti-γ-globulin factors. At a concentration of

TABLE I

Occurrence of Agglutinator Active against Red Cells Coated with Pepsin-Digested Anti-CD Ripley

Sera	+++ agglutination at dilution 1:5	++ or stronger agglutination at dilution 1:5	Total n
	per cent	*per cent*	
Blood donor sera	5	17	100
Mixed hospital population sera	10	20	140
Rheumatoid arthritis sera	45	66	60
Sera with Milgrom type anti-γ-globulin factors	100		5

1 mg/ml, γG-globulin aggregated by heating or treatment with mercaptoethanol and urea did not significantly inhibit the agglutination of red cells coated with pepsin-digested anti-CD Ripley.

Treatment of sera with mercaptoethanol gave various results. In some cases, complete loss of agglutinating ability was observed. In other cases, there was a reduction in titre or no effect. This is illustrated in Table II. These findings are different from those on rheumatoid factors and Milgrom type anti-γ-globulin factors which are included in the table for comparison. The latter two activities were uniformly destroyed by reduction.

Chromatography of selected sera showed that Milgrom type anti-γ-globulin factors and rheumatoid factor activity were only present in the fractions containing γM-globulin. Agglutinating activity against cells coated with pepsin-digested anti-CD Ripley could be recovered with fractions containing immunologically pure γG-globulin. It was also recovered with fraction 3 of serum En. (*cf.* Fig. 1). This fraction contained γA-globulin and only trace amounts of γG-globulin which could not account for the activity. Finally, this type of activity was recovered in fractions containing γM-globulin. Thus agglutinating

activity against cells coated with pepsin-digested anti-CD Ripley was found in all three classes of immunoglobulins. These observations confirm and extend previous density gradient ultracentrifugation experiments which revealed the activity in both the 7S and 19S fraction of human sera (8).

Comparison of Various Anti–γ-Globulin Factors by Absorption Experiments.— Absorption experiments were made to compare the reactivity of the three different types of anti–γ-globulin factors:

TABLE II

Effect of Reduction by Mercaptoethanol (ME) on Various Anti–γ-Globulin Factors

Serum	Rheumatoid factor*		Milgrom type‡ anti–γ-globulin factor		Agglutination of cells coated with pepsin-digested anti-CD Ripley	
	control	ME	control	ME	control	ME
Ja.	512	<2	Neg.§		32	4
Li.	128	<2	"		16	8
Je.	64	<2	"		64	32
Gu.	128	<2	"		8	8
He.	Neg. ‖		64	<2	64	<2
Ni.	"		32	<2	16	<2
Be.	"		32	<2	64	2
En.	"		32	<2	64	16
Sv.	"		32	<2	64	32

* Waaler-Rose test.

‡ Agglutination of red cells coated with anti-Rh Lo.

§ No agglutination at dilution 1:2 or higher.

‖ No agglutination at dilution 1:8 or higher.

First, antigen-antibody precipitate containing *rabbit* antibody (egg albumin/rabbit anti–egg albumin) was used for absorption. The results are summarized in Table III. As expected (3, 4) activity in the Waaler-Rose test was markedly reduced by this procedure, whereas activity in the latex fixation test was only slightly reduced. Milgrom type anti–γ-globulin factor activity and agglutination of red cells coated with pepsin-digested antibody remained unchanged.

Secondly, precipitate containing *human* antibody (diphtheria toxoid/human antitoxin) was used. In this instance, the activity of rheumatoid sera in both the Waaler-Rose and latex fixation tests was markedly reduced. Milgrom type anti–γ-globulin factor activity could also be absorbed with this antigen-antibody precipitate. Repeated experiments on a variety of sera showed that the agglutinating activity against cells coated with pepsin-digested anti-CD Ripley was easily removed by absorption with this type of antigen-antibody complex. As may be seen from Table IV, this was observed with sera that con-

TABLE III

Absorption Experiments with Antigen-Antibody Precipitates Containing Rabbit Antibody (Egg Albumin/Rabbit Anti-Egg Albumin)

Serum	Rheumatoid factors				Milgrom type anti-γ-globulin factor		Agglutination of cells coated with pepsin-digested anti-CD Ripley	
	Latex fixation test		Waaler-Rose test					
	Control	Absorbed	Control	Absorbed	Control	Absorbed	Control	Absorbed
Dr.	2560	2560	320	20	—		128	64
Jo.	2560	640	640	40	—		64	32
9486	Neg.*		Neg.‡		Neg.‡		32	16
9964	"		"		"		32	16
En.	Neg.		Neg.		64	64	32	32
Ni.	"		"		16	16	16	16
Sv.	"		"		32	16	32	32

* Negative at dilution 1:10 or higher

‡ Negative as defined in Table II.

TABLE IV

Absorption Experiments with Antigen-Antibody Precipitates Containing Human Antibody (Diphtheria Toxoid/Human Antitoxin)

Serum	Rheumatoid factors				Milgrom type anti-γ-globulin factor		Agglutination of cells coated with pepsin-digested anti-CD Ripley	
	Latex fixation test		Waaler-Rose test					
	Control	Absorbed	Control	Absorbed	Control	Absorbed	Control	Absorbed
Dr.	2000	40	512	64	—		256	<2
Jo.	4000	40	512	64	—		64	<2
99	Neg.*		Neg.*		Neg.*		32	<2
251	"		"		"		64	2
553	"		"		"		64	<2
680	"		"		"		32	<2
9486	"		"		"		64	<2
9964	"		"		"		64	2
En.	Neg.		Neg.		32	<2	32	<2
Ni.	"		"		32	<2	32	<2
Sv.	"		"		32	2	64	<2

* Negative as defined in Tables II and III.

tained only this type of anti-γ-globulin factor, and with sera that also contained other anti-γ-globulin factors. Inhibition experiments using similar, or many times greater, concentrations of diphtheria toxoid alone demonstrated that antigen alone had no inhibiting capacity. Thus it appears that the hidden antigen in human γG-globulin is revealed, not only by the somewhat artificial

laboratory conditions of enzymatic digestion at low pH, but also when human γG-globulin acts as antibody in an antigen-antibody precipitate.

Finally, absorption experiments were performed with antigen-antibody precipitates which contained *human* γG-globulin as *antigen* (human γG-globulin/rabbit anti-Fab). As shown in Table V, rheumatoid factors were easily absorbed by this type of antigen-antibody precipitate, whereas Milgrom type anti-γ-globulin factor activity and agglutination of red cells coated with pepsin-digested anti-CD Ripley remained unchanged. Additional control experiments showed that the difference in absorbing capacity of precipitates containing

TABLE V

Absorption Experiments with Antigen-Antibody Precipitates Containing Human γG-Globulin as Antigen (Human γG-Globulin/Rabbit Anti-Fab)

Serum	Rheumatoid factors				Milgrom type anti-γ-globulin		Agglutination of cells coated with pepsin-digested anti-CD Ripley	
	Latex fixation test		Waaler-Rose test					
	Control	Absorbed	Control	Absorbed	Control	Absorbed	Control	Absorbed
Dr.	2560	20	320	40	—		128	256
Jo.	5120	20	640	40	—		64	64
9486	Neg.*		Neg.*		Neg.*		32	32
9492	"		"		"		32	64
9964	"		"		"		32	64
En.	Neg.		Neg.		32	16	32	32
Ni.	"		"		32	16	32	64
Sv.	"		"		32	32	32	64

* Negative as defined in Tables II and III.

human γG-globulin as antibody and antigen respectively was not caused by differences in the amount of γG-globulin present in the two instances. These experiments confirm the inhibition experiments with aggregated human γG-globulin: whereas a variety of procedures which aggregate human γG-globulin make it reactive with rheumatoid factors, apparently more specific changes that occur when the γ-globulin molecule functions as antibody in an immune complex are needed to confer upon it ability to interact with the other two kinds of anti-γ-globulin factors.

DISCUSSION

It was observed independently by Grubb (9) and Milgrom *et al.* (21) that some human sera agglutinate red cells coated with incomplete anti-Rh antibodies, and that the agglutination is not inhibited by addition of γG-globulin. The active substance was called "anti-antibody" by Milgrom *et al.* since the serological properties indicated that it reacted with antibody γ-globulin only

when certain structural modifications had occurred as a result of its interaction with the corresponding antigen.

Various immunochemical data also indicate that structural modifications of the antibody molecule occur as a result of the antigen-antibody interaction: an increase in the apparent volume of the antibody molecules, similar to that obtained in protein denaturation by other means, has been observed (34, 35) as well as an increase in optical levorotation (36). Robert and Grabar (37) titrated SH groups in horse antibodies to human serum albumin and to *Salmonella gallinarum* polysaccharide. The antibodies in the antisera were partially purified and showed no titratable thiol groups. In the presence of antigen, however, there was a slow exposure of a small amount of titratable SH groups.

Autologous γ-globulin denaturated by various physicochemical procedures is antigenic (38–40), and the same is the case for autologous antigen-antibody complexes. Immunization with various forms of autologous antigen-antibody complexes leads to the formation of anti-γ-globulin antibodies with properties very similar to human rheumatoid factors (40–44).

The present experiments confirm that structural changes occur in antibody γ-globulin as a result of its interaction with the corresponding antigen. Osterland, Harboe, and Kunkel (8) detected in some human sera an agglutinating substance active against red cells coated with pepsin-digested incomplete anti-Rh antibodies. The agglutinator had many of the properties characteristic of antibodies and reacted with determinants on the Fab part of human γG-globulin that were hidden in the intact molecule and exposed by enzymatic digestion at low pH. The absorption experiments with specific precipitates consisting of diphtheria toxoid and human antitoxin (*cf.* Table IV) demonstrated that this hidden antigen is also exposed when human γG-globulin acts as *antibody* in an immune precipitate. The hidden antigen is, however, not exposed when γ-globulin is present as *antigen* in the precipitate (*cf.* Table V).

The hidden antigen is exposed on γG-globulin molecules which function as antibody in an immune precipitate, whereas the agglutination experiments show that it is not exposed on anti-Rh γG-globulin molecules attached to red cells. This suggests that sufficient molecular alteration of the γG-molecule to expose the antigen occurs only when lattice formation takes place. Similar observations have been made with Milgrom type anti-γ-globulin factors in rabbit sera (45). These do not interact with non-precipitating complexes of univalent or bivalent hapten with antihapten antibody (anti-DNP) but reacted with the precipitating system of anti-DNP–DNP–BSA.

Agglutination experiments using red cells coated with intact and pepsin-digested incomplete anti-Rh antibodies are useful to study the interaction of agglutinating factors with distinct parts of the anti-Rh γ-globulin molecule (8). In the present five cases, such experiments were complicated because the Milgrom type anti-γ-globulin factors were associated with potent agglutinators reacting with the hidden antigen on the Fab part of human γG-globulin.

Inhibition experiments indicated that the two substances were distinct. In one instance, a complete separation of the two substances was obtained by DEAE cellulose chromatography (*cf.* Fig. 1). The fraction which contained a Milgrom type anti–γ-globulin factor agglutinated red cells coated with whole anti-CD Ripley and other incomplete anti-Rh antibodies. It did not agglutinate cells coated with pepsin-digested anti-Rh, thus indicating that the active substance reacted with the Fc part of the anti-Rh molecules.

In the original paper, Milgrom *et al.* (21) showed that their anti–γ-globulin factors agglutinated red cells sensitized with a variety of incomplete antibodies (anti-D, anti-C, and some samples of anti-K and anti-Fy^a), whereas no reactivity was observed with anti-A and anti-B. In view of the known structural relationship between the various immunoglobulins (32, 33), reactivity of this agglutinator with various molecular forms of human antibodies would provide important evidence for its site of action. In the present experiments, various γG-globulin antibodies (anti-Rh, anti-A, and anti-B) were found to react with Milgrom type anti–γ-globulin factors, whereas no interaction with γM-globulin antibodies could be detected in experiments with anti-A, anti-B, and anti-*Salmonella* O. These findings also indicate that Milgrom type anti–γ-globulin factors in human sera react with the Fc part of γG-globulin since this part gives the molecule its distinctive properties.

Similar anti–γ-globulin factors in rabbit sera have been reported to react with the Fab fragment of the γG-globulin molecule (45, 46). The basis of this difference in behavior in the human and rabbit species is at present unknown.

The inhibition experiments with γG-globulin aggregated by heat or treatment with mercaptoethanol and urea showed that these materials reacted strongly with rheumatoid factors, whereas reactivity with the Milgrom type anti–γ-globulin factors could not be demonstrated. In experiments with similar rabbit sera (45, 46) some inhibition was obtained by γ-globulin aggregated by various techniques, but it was apparent that large amounts of aggregates had to be used to detect a rather weak inhibition. The structural changes in the γ-globulin molecule that are required to induce reactivity with Milgrom type anti–γ-globulin factors thus appear to be highly specific, whereas the changes required to increase the reactivity with rheumatoid factors can be more easily achieved. This is also indicated by absorption experiments using specific precipitates containing human γG-globulin as antigen and antibody respectively. Both types of complexes readily absorbed rheumatoid factors, whereas only the latter type was able to absorb Milgrom type anti–γ-globulin factors.

The three types of human anti–γ-globulin factors discussed presently are clearly distinguishable by inhibition experiments and in physicochemical properties. Rheumatoid factors detected by the usual serological procedures are γM-globulins (1, 2) and the same was the case for the present five Milgrom type anti–γ-globulin factors. The agglutinator active against cells coated with pepsin-digested antibodies is more diverse with regard to protein character. It

has previously been demonstrated in both the 7S and 19S fractions of serum by density gradient ultracentrifugation (8). In the present experiments, the activity was found in all three major classes of immunoglobulins. The diversity in physicochemical properties of various forms of anti–γ-globulin factors is an intriguing and as yet unexplained phenomenon.

The agglutinator active against red cells coated with pepsin-digested antibodies occurs rarely in normal subjects, more frequently in patients with various diseases and with a high frequency in rheumatoid arthritis (*cf.* Table I). Very striking was the observation that strong agglutinators of this kind occurred in all five sera that contained Milgrom type anti–γ-globulin factors. This finding may point to a common stimulus for their formation. Experimental (40–44) and clinical observations (47, 48) indicate that rheumatoid factors may be produced as a result of stimulation by autologous antigen-antibody complexes. Other anti–γ-globulin factors with somewhat different serological properties may also be the result of such stimulation. This mechanism appears particularly relevant with regard to the agglutinator active against the hidden antigen in human γG-globulin since the absorption experiments showed that this antigen also is revealed when human γG-globulin acts as antibody in an antigen-antibody precipitate.

It is, however, also possible that enzymatic unmasking of the antigen may occur *in vivo* in some cases since it has been shown that the intracellular pH during phagocytosis may be as low as 3.5 (49). By immunization with pepsin-split autologous γG-globulin, rabbits readily produce anti–γ-globulin antibodies (40).

SUMMARY

The serological and physicochemical properties of the following three forms of human anti–γ-globulin factors were compared: (*a*) rheumatoid factors; (*b*) Milgrom type anti–γ-globulin factors; and (*c*) factors directed against an antigen in human γG-globulin that is hidden in the intact molecule and revealed by enzymatic digestion at low pH. The property common to these factors is ability to interact with human γG-globulin; they are distinguishable because they react with different antigenic groups on this molecule.

In all of five sera, the Milgrom type anti–γ-globulin factors were γM-globulins. They reacted with various human γG-globulin antibodies but failed to interact with γM-globulin type antibodies in agglutination and absorption experiments. When isolated from other anti–γ-globulin factors, they agglutinated red cells coated with intact anti-Rh antibodies, but failed to react with cells cells coated with pepsin-digested anti-Rh antibody. These observations indicate that the agglutinator reacts with the crystallizable, inert fragment of γG-globulin.

Anti–γ-globulin activity directed against an antigen in human γG-globulin revealed by pepsin digestion was demonstrated in γG-, γA-, and γM-globulins.

This anti–γ-globulin factor could be absorbed by antigen-antibody precipitates containing human antibody, which shows that the hidden antigen in human γG-globulin is revealed not only by enzymatic digestion at low pH, but also when γG-globulin is present as antibody in an antigen-antibody precipitate. Rheumatoid factors and Milgrom type anti–γ-globulin factors were also absorbed by antigen-antibody precipitates containing human antibody.

The results indicate that the three distinct forms of anti–γ-globulin factors may all be produced as a result of antigenic stimulation by autologous antigen-antibody complexes.

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