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Detection of circulating antigen–antibody complexes by their inhibitory effect on the agglutination of IgG-coated particles by rheumatoid factor or C1q

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SUMMARY

The agglutination of Ig-coated particles by human RF or C1q can be inhibited by Ig aggregates or AgAb complexes.

The effect of Ig class was studied by means of agarose-linked human monoclonal Igs. RF was inhibited by all subclasses of IgG and IgA but not by IgM, whereas C1q reacted with IgM, IgG3 and IgG1. Heat-aggregated IgG3 was fractionated by gel-filtration on Ultrogel. Inhibition was restricted to certain fractions of aggregates, viz $(IgG3)_{\sim 7}$ and $(IgG3)_{\sim 21}$ for RF, and $(IgG3)_{\sim 10}$, $(IgG3)_{\sim 14}$ and $(IgG3)_{\sim 27}$ for C1q. In a precipitin curve experiment, it was found that RF was inhibited by soluble complexes over an extended range of AgAb ratios, the inactivation of C1q being limited to complexes with 2–5 times antigen excess.

Inhibiting factors were found in patients with various diseases and, at low titres, in 22% of healthy people.

In 27% of patients' sera, the inhibitors were demonstrable by C1q only after removal of endogenous RF by adsorption on insolubilized IgG. In several patients endogenous agglutinating activity and direct inhibitory activity tended to alternate during the course of the disease.

Sera from various patients were also filtrated on Ultrogel and the elution was monitored by immunoassay of IgA, IgM and IgG, as well as by the two inhibition tests. The inhibiting factors were distributed over several peaks which only partially coincided with the elution profiles of IgG and IgM.

INTRODUCTION

In 1961 Kunkel *et al.* described the inactivation by certain human sera of the agglutinating activity of rheumatoid factor (RF) on Rh erythrocytes coated with anti-Rh antibodies. Such sera proved to contain fast-sedimenting material when analysed in the analytical ultracentrifuge. The principle of agglutination-inhibition has also been applied to the detection of IgG complexes in eluates from rheumatoid tissue by Munthe & Natvig (1971) who used Ig-coated polystyrene particles (latex) as a substrate for the activity of RF. This latex technique for detecting immune complexes has appeared to us particularly simple in comparison with methods requiring the handling of delicate biological material such as Raji cells (Theophilopoulos *et al.*, 1974) or platelets (Penttinen, Vaheri & Myllylä, 1971), and/or the use of radioactive markers (Nydegger *et al.*, 1974; Ludwig & Cusumano, 1974; Cowdery, Treadwell & Fritz, 1975).

The present work describes a routine version of the method, which includes the novel feature that each inhibition assay is carried out in parallel with RF and C1q as the agglutinating agents. It will be shown that analytical combination enlarges both the sensitivity and the information content of the test. This, as will appear from our data, is due largely to the fact that the reaction of AgAb complexes with RF and

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C1q is markedly influenced both by the nature of the immunoglobulins involved and by the size of these AgAb complexes.

The tests we propose not only enable the detection of circulating immune complexes in all types of disorders where they have previously been reported to occur, but also in a variety of conditions—including some benign ones—where the presence of such complexes was suspected but not demonstrated. In addition our data emphasize the frequent association between the presence of such complexes and the production of rheumatoid factor.

MATERIALS AND METHODS

Sera. Sera from healthy individuals and patients were in general tested within 24 hr after collection. Some were kept for 4–5 weeks at 4°C or 6 months at −20°C and retested. The inhibition titre toward C1q or RF never increased upon storage. A slight decrease was observed in certain samples. A pool of normal sera which had been kept for more than a year at −20°C was found to be completely devoid of agglutinating or inhibiting properties.

Rheumatoid factor. Three RF-containing reagents were used: (i) whole rheumatoid arthritis serum; (ii) human RF purified by adsorption on insolubilized human IgG; and (iii) a fraction rich in human RF corresponding to the excluded portion of a Sephadex G-200 chromatography of the euglobulins of rheumatoid serum. For the three preparations, serum from a single patient with a titre of 1/320 in the latex test was chosen.

To prepare reagent (ii), IgG was isolated from normal human serum by DEAE–Sephadex chromatography and was insolubilized by conjugation to aminated Sepharose-4B by means of glutaraldehyde (Cambiaso *et al.*, 1975), at a ratio of 10 mg IgG per ml of packed beads. After adsorption of RF and washing with 1 M saline, RF was eluted by 0·75 ammonium thiocyanate and used after dialysis against 0·9% saline.

No clear difference was noted in the sensitivity of the inhibition agglutination tests performed with the three reagents. However, the titre of RF in whole rheumatoid serum tended to decrease with time, whereas purified fractions were found to be more stable. After several experiments, preference was given to the preparation obtained by gel-filtration because the yield was better with this procedure than with the adsorption technique. RF preparations were used after appropriate dilutions with 0·9% saline containing 0·1 M glycine adjusted at pH 8·2.

C1q. This was isolated from fresh serum by the technique of Yonemasu & Stroud (1971). The reagent was stored at a concentration of about 1 mg/ml at 4°C in saline. Prior to use, C1q was diluted in saline buffered by 0·1 M glycine at pH 8·2 containing 30 mg/ml bovine serum albumin and 10mM Ca^{2+}.

Addition of EDTA–sodium to C1q significantly increases its agglutinating activity. However, the chelator from the reaction medium releases C1q from C1 present in the serum to be tested. This endogenous C1q, by adjuncting itself to the C1q employed as the reagent, is liable to decrease the sensitivity of the test. Inactivation of the endogenous C1q by heating the serum at 56°C for 30 min prevents this sort of interference. However, it has been noted that several samples which displayed a clear inhibiting activity lost this property after heating, suggesting that increase in temperature could cause an irreversible dissociation of the AgAb complexes. Therefore, in our routine assay, we did not heat the serum and C1q was used without adding EDTA.

It was also observed that certain sera or aggregates of IgG which were capable of inhibiting C1q lost this property when chromatographed on Sephadex G-200. When Ca^{2+} was added to C1q (10 mM), inhibitory activity was restored. This phenomenon is probably related to the stabilizing effect of Ca^{2+} on certain AgAb complexes and Ig aggregates, as will be shown in a later publication (Cambiaso *et al.*, in preparation). These observations prompted us systematically to include 10 mM Ca^{2+} in the C1q reaction mixture.

Immunoglobulins. Normal IgG was isolated from a pool of human sera by DEAE–Sephadex chromatography, and was aggregated, at a concentration of 10 mg/ml in saline, by heating at 63°C for 10 min (Müller-Eberhard & Kunkel, 1961).

Monoclonal human IgG2 and IgG4 were kindly provided by Dr H. G. Kunkel (Rockefeller University, New York), Dr J. B. Natvig (Oslo), Dr J. R. Hobbs (London) and Dr M. Seligmann (Paris). Dr J. P. Vaerman (Brussels) gave us samples of monoclonal human IgA1 and IgA2 in their monomeric and polymeric forms, as well as monoclonal human IgM, IgG1 and IgG3. Human serum albumin (HSA) was purchased from Behring Institute (Marburg, West Germany).

All the proteins were insolubilized by conjugation to aminated agarose beads by means of glutaraldehyde (Cambiaso *et al.*, 1975).

Deoxyribonucleic acid. Calf thymus deoxyribonucleic acid (DNA) was obtained from Schwarz Laboratories (Mount Vernon, New York). Single-stranded DNA was prepared by heating the native DNA at 100°C for 10 min and immediate cooling in an ice bath.

Fractions from gel-filtration of patients' sera were digested by DNase I (Worthington Biochemical Corp., New Jersey) at pH 7·4 (25 μM Tris buffer containing 10 mM magnesium chloride) and an enzyme concentration of 50 μg/ml, for 2 hr at 37°C.

The inhibition test. The agglutination was always performed by the same person, using human IgG-coated polystyrene particles (Latex-RF-Reagenz) purchased from Behring Institute (Marburg, West Germany). Samples of 25 μl of the RF or C1q solutions to be described below were mixed on a dark plate with an equal volume of the sample and then with 25 μl

of the latex suspension. During the following 3-min interval the plate was inspected repeatedly, and agglutinations were scored as either positive or negative.

For optimal sensitivity, C1q and RF were employed at their lowest concentrations still causing distinct agglutination, and these concentrations were determined anew for each series of tests.

The inhibiting activity of the samples was titrated by two-fold serial dilution of the latter.

When observed, the interference by endogenous RF in certain patients' sera was dealt with by adding dithiothreitol (DTT) to such samples at a final concentration of 1·3 mM. This step effectively suppressed agglutinating activity of endogenous RF within 5 min. To destroy residual DTT which otherwise would react with the agglutinating agent—whether RF or C1q—it was found sufficient to expose the reduced sample to atmospheric oxygen by simply leaving the test tube open for 45 min.

An alternative method to remove endogenous agglutinating activity was to pass the sample through a small column of IgG–agarose conjugates (0·1 ml of serum per 0·2 ml of packed gel). The latter procedure takes only 10 min and is very easy to perform, so that it has now become routine practice at our laboratory.

Precipitin curve. Increasing amounts (0·2–30 mg) of tetanus toxoid (Institut Mérieux, Lyon, France), in a volume of 0·4 ml of 0·9% saline were added to 0·2 ml of a commercial preparation of human IgG rich in anti-tetanus antibodies (Tetaglobuline, Institut Mérieux, France).

After incubation at 4°C for 1 week, the precipitates were washed twice with 0·9% saline at 4°C, dissolved in 0·2 ml of 0·1 M sodium hydroxide, and protein was determined by the Folin–Ciocalteu method. The supernatants were tested for their inhibitory activity on C1q and RF.

Gel-filtration. Gel-filtration was performed in a column (1 m × 2·5 cm) of Ultrogel AcA 22 (LKB, Bromma, Sweden) with a flow rate of 10 ml/hr. The column was calibrated with human serum, the elution of IgA, IgG, α_2-macroglobulin and IgM being monitored by automated immunoassays (AIP system, Technicon, Tarrytown, New York). Horse ferritin (Koch-Light, Colnbrook, England), human thyroglobulin (kindly provided by Dr P. De Nayer, Brussels) and dimers of a human monoclonal IgM protein (a gift of Dr J. P. Vaerman, Brussels) were used as additional markers.

Immunoassay of serum proteins. Serum proteins were determined by an automated nephelometric immunoassay (AIP system, Technicon, Tarrytown, New York). The specific antisera were all of goat origin. For most proteins, reliable standards were not available. The results are therefore given as a percentage of a reference serum pool prepared from a thousand blood donors.

In the section of Results dealing with the correlation between the level of C3 and the occurrence of inhibiting or agglutinating factors, C3 was considered as significantly decreased when its concentration did not exceed 60% of that of the serum pool. Such a change corresponds to a decrease of at least two standard deviations (Ganrot, 1972). However, since the production or release of C3 increases during inflammation, its overconsumption may be masked in certain diseases. Therefore, we have taken into account the influence of possible phlogogenic stimuli by measuring the concentration of another acute phase reactant, viz α_1-antitrypsin. The latter is more sensitive to inflammation than is C3. During an inflammatory reaction not due to an immune process, for instance a surgical trauma, the α_1-antitrypsin/C3 ratio calculated from concentrations expressed in per cent of a serum pool should not exceed 1·8 (Aronsen *et al.*, 1972). Hence, when the plasma-protein spectrum pointed to an inflammatory condition, the consumption of C3 was considered as abnormally high if the α_1-antitrypsin:C3 ratio was equal or larger than 1·8.

RESULTS

Inhibition by various agarose-linked Igs—influence of classes and subclasses

It is difficult to prepare AgAb complexes involving human antibodies belonging to selected Ig classes and subclasses. Hence, the study of the influence of the antibody nature on the reaction of complexes with C1q and RF was carried out with artificially polymerized Ig. For this purpose various monoclonal Ig, as well as HSA used as a control, were conjugated to agarose beads.

Solutions of pure RF or C1q (0·5 ml), able to agglutinate latex at dilutions of 1/128 and 1/16 respectively, were incubated for 30 min at room temperature with suspensions of Ig- or HSA-coated agarose (0·25 ml of packed gel). The supernatants were then tested for residual agglutinating activity (Table 1). Clear inhibition of C1q was observed with insolubilized IgG3 and IgM, whereas only a slight decrease in agglutination titre was noted with IgG1. As regards RF, all IgG and IgA but not IgM displayed marked inhibitory properties.

Inhibition by IgG3 aggregates of different sizes

Human IgG3 (10 mg/ml), aggregated by heat, was filtrated on a column of Ultrogel equilibrated with 0·9% saline. Elution was monitored by absorbance at 280 nm and by inhibition of C1q or RF (Fig. 1). Aggregated IgG3 resolved into two major peaks corresponding to polymers. No protein was eluted in the region where monomers were expected. C1q was inhibited mainly by aggregates consisting of about

TABLE 1. Agglutinating activity (dilution titres) of human C1q or RF after incubation with various human monoclonal Igs or human serum albumin linked to agarose*

	C1q	RF
Initial titre	1/16	1/128
Titre remaining after incubation with linked:		
Albumin (control)	1/16	1/128
IgG1	1/8	1/32
IgG2	1/16	1/32
IgG3	1/1	1/16
IgG4	1/16	1/32
IgA1 monomer	1/16	1/32
IgA1 polymer	1/16	1/16
IgA2 monomer	1/16	1/16
IgA2 polymer	1/16	1/16
IgM	1/1	1/128

* 0·5 ml of C1q or RF solution absorbed with 0·25 ml of packed agarose carrying 1·2 mg of covalently linked protein.

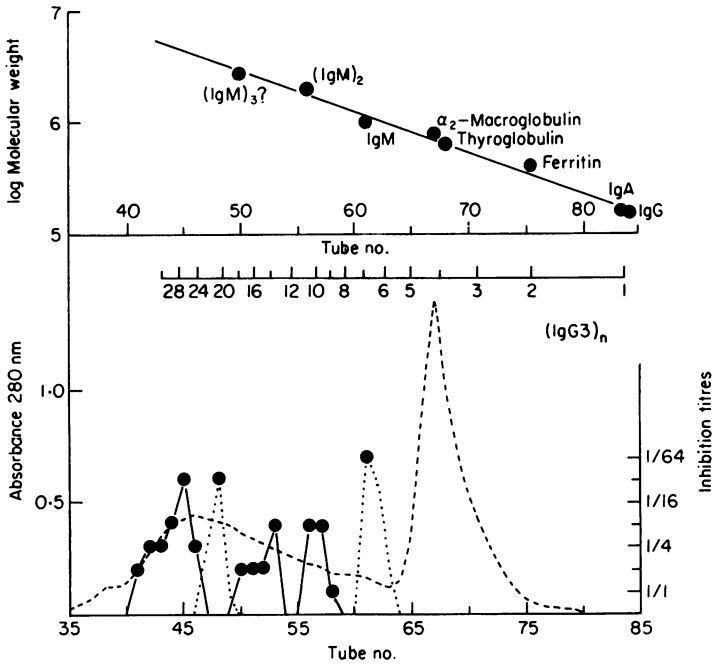

FIG. 1. Gel-filtration of heat-aggregated human monoclonal IgG3 on Ultrogel (below). Elution was monitored by absorbance at 280 nm (– – –) and inhibition of the agglutination of IgG-coated particles by RF (·····) and C1q (——). The column was calibrated (above) by immunoassay of various proteins in the eluate from a sample of human serum, i.e. IgG (mol. wt = $1{\cdot}53\times10^5$), monomeric IgA (mol. wt = $1{\cdot}62\times10^5$), α_2-macroglobulin (mol. wt = $7{\cdot}6\times10^5$), and IgM which resolved into a major peak corresponding to monomeric IgM (mol. wt = $9{\cdot}5\times10^5$) with two small shoulders in its ascending part, presumably representing dimeric and trimeric IgM. Purified proteins were also used for calibration, i.e. monoclonal, dimeric, human IgM (mol. wt = 19×10^5), human thyroglobulin (mol. wt = $4{\cdot}4\times10^5$). From these data and on the basis of a mol. wt of $1{\cdot}72\times10^5$ for monomeric human IgG3 (Saluk & Clem, 1971), the scale for the particle size of aggregated IgG3 was constructed (below).

TABLE 2. Minimal concentrations (in μg/ml) of different aggregates of IgG3 detectable by the C1q- or RF-inhibition tests

Reagents	$(IgG3)_{\sim 27}$*	$(IgG3)_{\sim 14}$	$(IgG3)_{\sim 10}$	$(IgG3)_{\sim 10}$	$(IgG3)_{\sim 7}$
C1q	10	> 300	26	20	> 120
RF	> 300	10	> 210	> 160	2

* Subscripts indicate the average number of monomeric IgG3 units (as determined by gel-filtration) forming the heat-produced aggregates of monoclonal human IgG3.

twenty-seven molecules of IgG3 (for notation of symbols see footnote to Table 2). Slight inhibition was also observed with fractions corresponding to $(IgG3)_{\sim 14}$ and $(IgG3)_{\sim 10}$. RF was inhibited by aggregates of $(IgG3)_{\sim 7}$ and by $(IgG3)_{\sim 21}$.

On the basis of the absorbance at 280 nm ($E^{1\%}_{280nm,\ 1cm}$ = 13·8) the concentration of IgG3 was estimated in the chromatographic fractions inhibiting C1q or RF. The absorbance was corrected for turbidity due to aggregation by the method of Wetlaufer (1962). Taking into account the maximal dilution at which the fractions were still able to inactivate each agglutinating reagent, a threshold of sensitivity, expressed in micrograms of IgG3 per millilitre, was calculated for various sizes of aggregates (Table 2). The minimum concentration of proteins detected was 2 μg/ml of the specific aggregate $(IgG3)_{\sim 7}$ by RF compared with 10 μg/ml of the aggregate $(IgG3)_{\sim 27}$ with C1q.

When the sensitivities of RF and C1q were tested with whole IgG purified from normal serum by chromatography on DEAE–Sephadex and aggregated by heat, C1q was inhibited by an IgG concentration of 26 μg/ml, whereas a concentration of about 140 μg/ml of the same preparation was required for absorbing RF.

Inhibition by AgAb complexes—influence of the AgAb ratio

AgAb complexes were prepared by the addition of increasing amounts of tetanus toxoid to a constant amount of an immunoglobulin preparation rich in anti-tetanus antibodies. After discarding the precipitates by centrifugation, the supernatants were tested for their inhibiting capacities towards C1q and RF (Fig. 2). Maximal inhibition of C1q occurred in the zone of two-fold antigen excess, whereas RF was

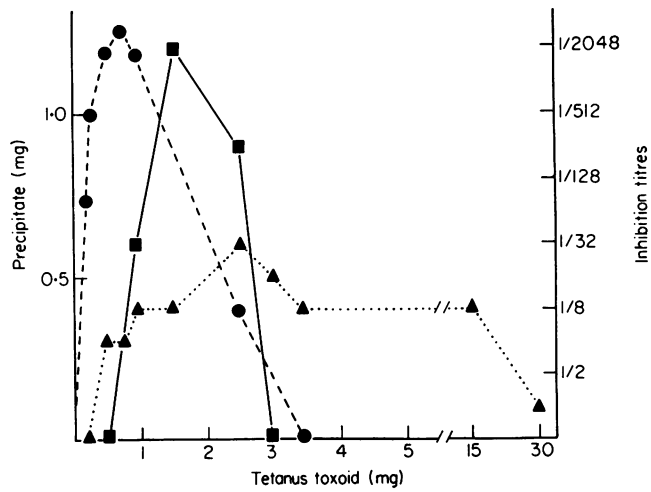

FIG. 2. Inhibition of the agglutination of IgG-coated polystyrene particles by C1q (——) and RF (·····) by the supernatants of precipitates (– – –) obtained by the addition of increasing amounts of tetanus toxoid to human immunoglobulins rich in anti-tetanus antibodies.

inhibited over a large range of Ag/Ab ratios extending from the equivalence point to a thirty-fold antigen excess with a maximum at three to four times antigen excess.

Inhibition by AgAb complexes—influence of size

Sera from patients with immune complex diseases such as thrombocytopenia, ankylosing spondylitis, systemic lupus erythematosus, and Crohn's ileitis were fractionated by gel-filtration on Ultrogel and the elution was monitored by absorbance at 280 nm, by inhibition of RF and C1q, as well as by immunoassays of IgG, IgA and IgM.

A typical elution pattern of the serum from a patient with ankylosing spondylitis and glomerulonephritis is presented in Fig. 3. This serum did not contain any detectable endogenous RF. Material

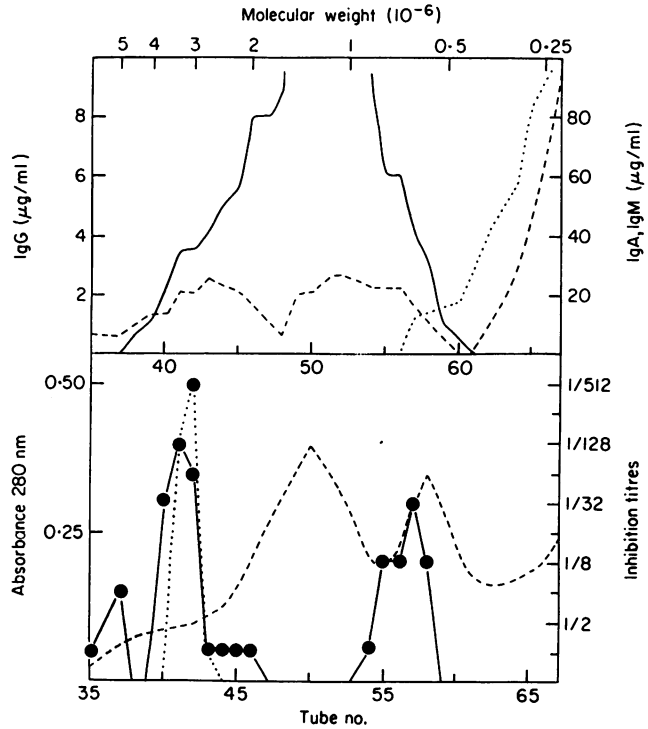

FIG. 3. Gel-filtration of the serum (3 ml) from a patient with ankylosing spondylitis and glomerulonephritis. The elution (fractions of 5 ml) was monitored by absorbance at 280 nm (– – –) and inhibition of the agglutination of IgG-coated polystyrene particles by C1q (——) and RF (·····) (lower graph), as well as by immunoassays of IgG (– – –), IgA (·····) and IgM (——) (upper graph). The column was calibrated as indicated in Fig. 1. The elution diagram is restricted to proteins with a molecular size exceeding 150,000.

inhibiting RF was gathered in a single peak corresponding to an approximate molecular size of $3{\cdot}0 \times 10^6$ Daltons. In contrast, material inhibiting C1q was found scattered over at least three major peaks with mean molecular sizes of, respectively, 5×10^6, $3{\cdot}3 \times 10^6$ and $0{\cdot}63 \times 10^6$ Daltons. None of these materials appeared to contain IgA (threshold of detection $\simeq$ 2·5 μg/ml). Both IgG and, to a lesser extent, IgM, were found throughout the molecular size range encompassing the inhibitory factors. IgG showed two ill-defined maxima, with mean molecular sizes of respectively, $2{\cdot}7 \times 10^6$ and $1{\cdot}0 \times 10^6$ Daltons, whereas the ascending slope of the IgM distribution showed two small shoulders possibly corresponding to, respectively, the dimer and trimer of the 19S unit. The single zone of RF-inhibiting activity vaguely matched with both the hypothetical IgM trimer shoulder and the $2{\cdot}7 \times 10^6$, peak of IgG. It was not possible to assign the different C1q-inhibiting factors to any of the IgG or IgM populations. A striking finding was the apparent absence of inhibitory activity of any specificity in the heavier part of the $1{\cdot}0 \times 10^6$ IgG peak.

Equally complex elution patterns were obtained with the other pathological sera. In general, both the C1q-inhibiting activity and the RF-inhibiting activity were distributed over several peaks, with a tendency for the former to be localized in the heaviest fractions.

When sera from patients with idiopathic thrombocytopenia were filtrated on Ultrogel, DNA could be identified in the fractions eluted with the void volume by its absorbance at 260 nm and by digestion with DNAase. Some of these fractions inhibited C1q but not RF, without showing any evidence for containing IgG (threshold 1 μg/ml) or IgM (threshold of detection ≃ 2·5 μg/ml). Reduction and alkylation of such samples, as recommended by Agnello, Winchester & Kunkel (1970), failed to suppress their inhibitory activity, suggesting that the factors involved were free single-stranded DNA rather than AgAb. To further explore this phenomenon, double-stranded (native) or single-stranded (heat-denatured) calf thymus DNA were dissolved at ten different concentrations (10–100 μg) in normal human serum and tested for their inhibitory activity on C1q or RF. Native DNA was found unable to inactivate either reagent, whereas single-stranded DNA at concentrations above 60 μg/ml clearly inhibited C1q without having any effect on RF.

Sera from healthy individuals

Sera from a hundred blood donors were analysed by the two agglutination tests. Twelve sera inhibited both C1q and RF at a titre of 1/1, whereas a further ten reacted with C1q at a 1/2 dilution. Two of the latter sera also inhibited RF at the same dilution. Circulating inhibiting factors may therefore be concluded to exist in one-fifth of the blood donor population, i.e. in a group of persons already selected for their lack of detectable pathology.

Five persons whose sera had at one time been found to give negative reactions with both tests, were submitted to a brief longitudinal study during the development of mild illnesses associated with sore throat, headache, tiredness or urticaria In none of these episodes was an aetiological diagnosis made. In every case, however, did inhibitory activity towards both RF and C1q appear at titres reaching from 1/1 to 1/64, only to disappear again after 1–7 days.

TABLE 3. Proportion of sera from patients with various diseases agglutinating IgG-coated particles or inhibiting their agglutination by RF or C1q

Diagnosis	No. of patients	Agglutinating sera	Inhibiting sera*			Non-agglutinating and non-inhibiting sera
			C1q only	RF only	Both C1q and RF	
Crohn's ileitis	66	1 (1·5%)	3 (4·5%)	4 (6%)	58 (88%)	0
Idiopathic thrombocytopenia	29	5 (17%)	0	0	24 (83%)	0
Chronic liver disease	19	13 (68%)	0	0	6 (32%)	0
Glomerulonephritis	13	4 (31%)	0	1 (7·5%)	7 (54%)	1 (7·5%)
Rheumatoid arthritis	14	13 (93%)	0	0	1 (7%)	0
Myelomatosis	14	3 (21·5%)	0	0	7 (50%)	4 (28·5%)
Leukaemia	14	5 (36%)	0	0	6 (43%)	3 (21%)
Acute lymphoid	(7)	(2)	0	0	(4)	(1)
Acute myeloid	(5)	(2)	0	0	(2)	(1)
Chronic myeloid	(2)	(1)	0	0	0	(1)
Lymphosarcoma	12	3 (25%)	0	0	9 (75%)	0
Ankylosing spondylitis	12	3 (25%)	0	0	9 (75%)	0
Systemic lupus erythematosus	9	4	0	0	5	0
Autoimmune haemolytic anaemia	8	5	0	0	3	0
Scleroderma	5	0	0	0	5	0
Chronic urticaria	5	0	0	0	5	0
	220	59 (27%)	3	5	145 (66%)	8 (4%)

* Frequencies of inhibition as obtained without steps being taken to disclose inhibitory activity masked by endogenous RF activity.

Sera from patients with various diseases

The inhibition tests were first applied to unselected samples without prior knowledge of the diagnosis. The investigations were then pursued on a restricted number of diseases, especially idiopathic thrombocytopenia and Crohn's disease.

Several samples (27%) were found to contain RF, particularly in chronic liver disease, autoimmune haemolytic anaemia, systemic lupus erythematosus, and rheumatoid arthritis (Table 3). Fifty-nine of these sera were treated with a reducing agent (DTT) in order to inactivate RF, and were tested again for their inhibiting activity. Twenty-four of them (40%) became able to inhibit C1q but not RF whereas the remaining sera retained a slight agglutinating activity interfering with the inhibition tests. The endogenous RF of twenty different sera were also neutralized by adsorption on insolubilized IgG. After such treatment, factors inhibiting C1q but not RF were detected in all samples. In most diseases mentioned in Table 1, except glomerulonephritis, leukaemia and myeloma, all sera displayed either agglutinating or inhibiting activity. Most often, the inhibiting factors were detected by both RF and C1q.

As regards the titres of inhibition, large variations were noted between patients having the same disease (Fig. 4).

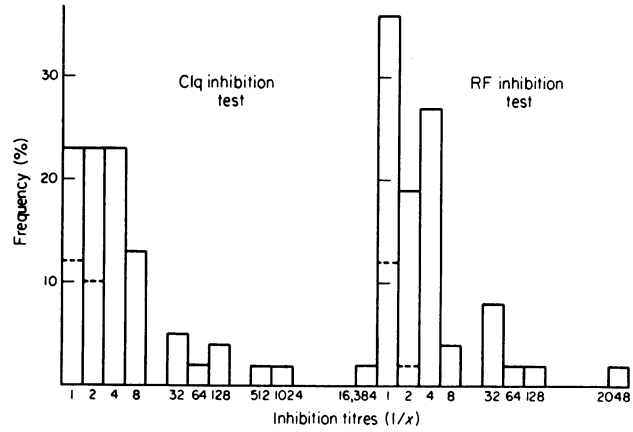

FIG. 4. Frequency distribution of inhibition titres towards C1q and RF in fifty-two patients with Crohn's disease (——) and a hundred healthy blood donors (– – –).

In general, the inhibition titres for C1q were higher than for RF. For C1q the range of inhibition titres extended, for instance, from 1/1 to 1/8000 in idiopathic thrombocytopenia, or from 1/1 to 1/16,000 in Crohn's disease, whereas for RF the inhibition titres never exceeded 1/64 in idiopathic thrombocytopenia and 1/128 in Crohn's disease.

Since circulating AgAb complexes are known to consume complement factors, especially C3, we made a study of the correlation between low levels of C3 and the presence of inhibiting or agglutinating factors in the sera from various patients. We have chosen 169 sera on the basis of the index defined under Materials and Methods, namely an α_1-antitrypsin/C3 ratio equal to or exceeding 1·8. It was found that 41% of these sera contained inhibiting factors, and that 38% displayed agglutinating activity. In a reciprocal survey, those sera that contained either inhibiting or agglutinating factors were analysed for their C3 content (Table 4). In certain diseases such as Crohn's ileitis, rheumatoid arthritis and ankylosing spondylitis, most sera had a normal concentration of C3, whereas in glomerulonephritis and chronic liver disease, C3 was low in almost 70% of the cases. A higher proportion of low C3 was found among agglutinating sera (56%) than among inhibiting sera (26%). However, this difference is not significant since within the population with low C3 described above, similar proportions of agglutinators and inhibitors were found.

TABLE 4. Proportion of sera with a low level of C3 in patients with circulating factors either inhibiting RF or C1q or agglutinating IgG-coated particles

Diagnosis	Low C3/inhibitors and/or agglutinators	Low C3/ agglutinators	Low C3/inhibitors		
			C1q only	RF only	Both C1q and RF
Crohn's ileitis	15/66 (23%)	0/1	1/3	2/4	12/58 (21%)
Idiopathic thrombocytopenia	4/22 (18%)	0/1	0	0	4/21 (19%)
Chronic liver diseases	13/19 (68%)	11/13 (85%)	0	0	2/6
Rheumatoid arthritis	3/14 (21%)	3/13 (23%)	0	0	0/1
Ankylosing spondylitis	2/12 (17%)	1/3	0	0	1/9
Glomerulonephritis	8/12 (67%)	3/4	0	1/1	4/7
Leukaemia	5/12 (42%)	3/6	0	0	2/6
Acute lymphoid	3/6	1/2	0	0	2/4
Acute myeloid	1/4	1/2	0	0	0/2
Chronic myeloid	1/2	1/2	0	0	0
Myelomatosis	5/10	1/3	0	0	4/7
Systemic lupus	5/9	3/4	0	0	2/5
Autoimmune haemolytic anaemia	4/8	4/5	0	0	0/3
	64/184 (35%)	29/53 (55%)	1/3	3/5	31/123 (25%)

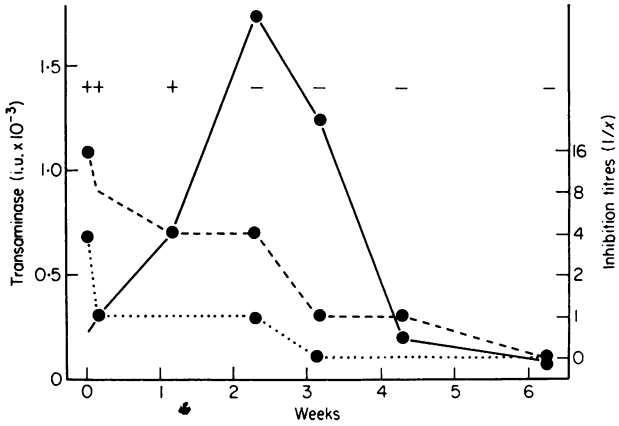

FIG. 5. Correlations between inhibitory activity towards C1q (– – –) and RF (·····), glutamic–pyruvic transaminase activity (——) and the presence (+) or absence (−) of Australia hepatitis antigen (as detected by counter-electrophoresis), during the evolution of a case of hepatitis B.

Longitudinal studies

The inhibition of C1q and RF was repeatedly titrated in sera collected during the evolution of diseases such as acute hepatitis B (Figs 5 and 6), idiopathic thrombocytopenia (Fig. 7) and Crohn's disease (Fig. 8).

In a first case of hepatitis B, inhibition of C1q and RF was already present at the onset of clinical symptoms. The titres progressively declined throughout the rise and fall of the peaks of glutamic–pyruvic and glutamic–oxaloacetic transaminase activity (not shown), and both types of inhibiting factors had vanished completely by the time these enzymes had returned to their normal level, i.e. by the sixth week. Hepatitis B antigen was detected by counter-electrophoresis during the 1st week. No endogenous agglutinating activity was detected at any time.

The second phase of hepatitis B was first brought to medical attention when the serum transaminase

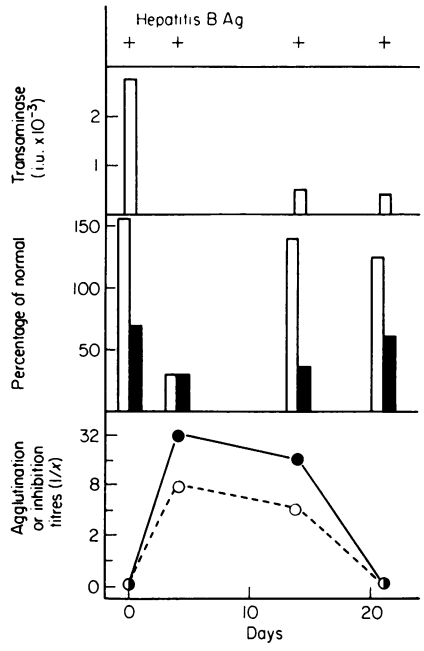

FIG. 6. Correlations between spontaneous agglutinating activity (●), inhibitory activity (○) towards C1q (after elimination of endogenous RF), concentrations of haptoglobin (□) and C3 (■) (as a percentage of their normal values in a pool from a thousand blood donors' sera), glutamic–pyruvic transaminase activity, and the presence or absence of Australia antigen (as detected by counter-electrophoresis), during the evolution of a case of hepatitis B.

levels were very high and presumably already on the decline. Endogenous agglutinating activity was not found at that moment, but became transiently detectable during the waning phase of the disease. After adsorption of the agglutinating factors on insolubilized IgG, these samples inhibited C1q but not RF. Concomitantly with the transient appearance of endogenous agglutinating activity, there occurred a drop in the serum levels of haptoglobin and C3. Both proteins re-entered the normal range during convalescence, the latter earlier so than the former.

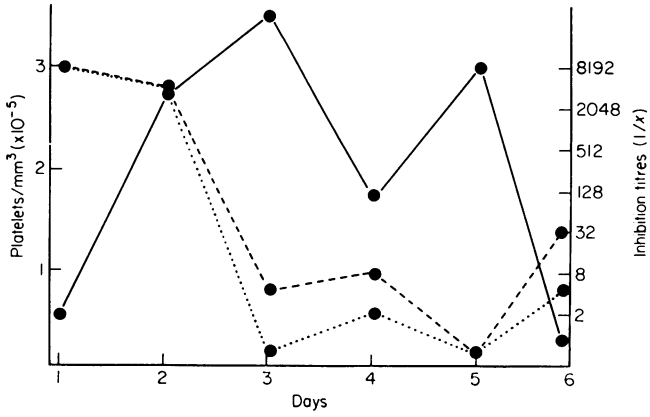

FIG. 7. Inhibitory activity towards C1q (– – –) and RF (·····), and platelet counts (——) during the evolution of a case of idiopathic thrombocytopenia.

In a case of idiopathic thrombocytopenia, the evolution of the platelet count was compared with that of the inhibition titres (Fig. 7). Broadly, there existed reciprocal relationship between the two, i.e. the number of platelets tended to decrease whenever inhibiting activity increased.

In a patient with Crohn's disease (Fig. 8), the evolution of the inhibition titres was studied over a 10-

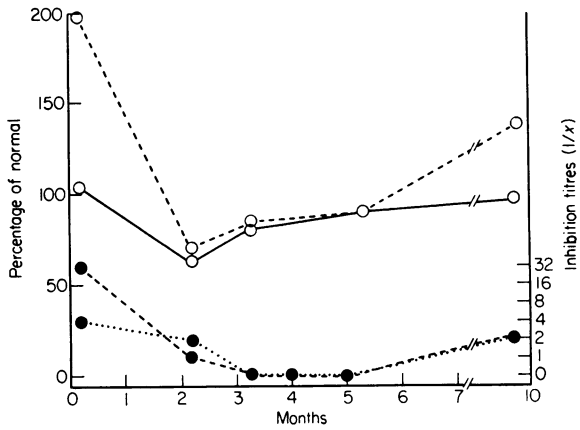

FIG. 8. Inhibitory activity towards C1q (● – – ●) and RF (● · · · ●), and concentrations of haptoglobin (○ – – ○) and C3 (○ · · · ○) as a percentage of their normal values in a pool of sera from a thousand blood donors) during the evolution of a case of Crohn's disease.

month period in parallel with the serum concentrations of haptoglobin and C3 used as indices of inflammation and consumption of complement, respectively. All this time the patient remained under glucocorticoid and salazopyrine therapy. Clinical symptoms were severe at the onset of the study. The inflammatory condition at that time was appropriately reflected by the high haptoglobin concentration, but not by that of C3 which remained within normal limits, indicating that complement was being consumed. This conclusion was substantiated by the finding of inhibitory activity towards both C1q and RF. As clinical improvement was brought on by therapy, both haptoglobin and C3 became normalized whereas inhibitory factors disappeared. The relapse that followed was associated with the reappearance of precisely the same abnormalities that existed at the beginning of the observation.

In four additional patients with Crohn's disease, as well as in two cases of ankylosing spondylitis and four other cases of idiopathic thrombocytopenia, inhibitory and endogenous agglutinating activities were found to alternate during the evolution of the disease.

The serum levels of immunoglobulins IgM and IgG never showed any obvious correlation with inhibitory activity, whether in longitudinal studies of individual patients or by comparison between different patients. Aggregate-free human IgG at a concentration of 10 mg/ml did not display any inhibitory activity on C1q or RF.

DISCUSSION

The present study demonstrates that agglutinating systems consisting of either a human RF or human C1q, on one hand, and human IgG-coated polystyrene particles on the other, are suitable for the detection, by inhibition, of immune complexes in human sera.

With RF as the agglutinating agent, the complexes detected are those that contain antibodies belonging to any subclass of IgG or IgA, whereas complexes of IgM apparently do not react in this system. The behaviour of IgD or IgE complexes in the RF system has not yet been explored. The occurrence of autoantibodies directed against classes of immunoglobulins other than IgG has been reported by Rivat, Rivat & Ropartz (1973) as well as by Williams *et al.* (1972), who have also shown that the spectrum of

these anti-Ig activities differs markedly from one patient to another. All our experiments with RF were carried out with material from a single rheumatoid patient, so that the general validity of our conclusions with this system remains to be verified.

With C1q as the agglutinating reagent, the highest inhibitions were obtained with complexes containing IgM or IgG3, followed by IgG1, whereas none of the other immunoglobulins appeared to react. Again, IgD and IgE remain to be tested. Although the IgG2 subclass is able to fix complement (Ishizaka *et al.*, 1967), it has less affinity for C1q than have either IgG3 or IgG1 (Müller-Eberhard, 1968). One may surmise that the density of IgG2 distribution on the agarose beads used by us was not adequate for the detection of inhibitory activity in this subclass.

In the precipitin curve experiment, C1q reacted with complexes confined to a restricted zone of antigen excess, just beyond the equivalence point. This agrees with the observation of Agnello *et al.* (1970) who found that the precipitin reaction between C1q and AgAb complexes occurred in the region of 2–5 times antigen excess. In our hands, RF was inhibited over an extended range of Ag/Ab ratios including the equivalence point and the zone of antibody excess, where soluble complexes would not be expected to occur. The presence of soluble complexes in this zone was presumably due to the involvement of low-affinity antibodies which, even if present in excess, were unable to precipitate their antigen.

Gel-filtration of IgG3 aggregates has shown that they must reach a critical particle size to become detectable by inhibition of agglutination, viz respectively $(IgG3)_{\sim 7}$ for RF, and $(IgG3)_{\sim 10}$ for C1q. It is clear, however, that a large size is not the only requirement to be met by an immunoglobulin aggregate to react with RF or C1q, since some of the IgG3 aggregates which exceeded the size thresholds defined above were inactive. Perhaps they did not possess a certain configuration required to allow C1q or RF to accede to their respective binding sites on the IgG3 molecules. This hypothesis would also account for the apparently erratic distribution of inhibitory activity along the chromatographic elution diagrams of individual patients' sera. Here the situation might, however, be more complex, since sera may contain different factors liable to interfere with the inhibition tests. For instance, single-stranded DNA, if present in sufficient amounts, may inactivate C1q in the absence of any anti-DNA antibody (Agnello *et al.*, 1970). Secondly, certain immune complexes may be saturated with endogenous RF and therefore be unable to neutralize exogenous RF employed as the agglutinating reagent. Such complexes might conversely be particularly active in the inhibition of C1q, as suggested by the fact that all agglutinating sera were found to contain C1q-inhibitory factors after elimination of endogenous RF. The binding of endogenous RF to immune complexes would explain why so many sera react with C1q even if the spectrum of sensitivity of C1q is much less extended than that of RF with respect both to the AgAb ratio and to classes or subclasses of antibody. Finally one has to consider the possibility of circulating complexes being saturated by the patient's own endogenous C1, as discussed by Johnson, Mowbray & Porter (1975). However, this would hardly be a major cause of interference with the C1q inhibition test, since the affinity of isolated C1q for immunoglobulins exceeds that of whole C1 (Müller-Eberhard, 1968), so that competitive displacement would be expected to take place during the test. This difference in reactivity is reflected, among other instances, by the precipitating (Müller-Eberhard & Kunkel, 1961; Taranta, Weiss & Franklin, 1961) and agglutinating (Ewald & Schubart, 1966) properties shown by free C1q but not by whole C1. This makes it understandable how AgAb complexes may coexist in the blood with normal levels of complement factors and yet be able strongly to react with C1q *in vitro*.

In 1956, Grubb discovered the Gm system by observing that certain sera inhibited the agglutinating activity of selected rheumatoid sera on Rh erythrocytes coated with anti-Rh antibodies. The antibody activity of our RF with respect of its anti-Gm activity is not known but the interference of Gm group in our study is unlikely since the inhibitory activity of 98% of sera concerned both RF and C1q. For the same reason, non-antibody bound single-stranded DNA which does not react with RF plays a negligible role, if any, in the patients' sera. However, among the 22% of normal sera with weak inhibitory capacity such activity was in one-half of the cases restricted towards C1q.

Repeated freezing and thawing of sera can perhaps induce the formation of IgG aggregates, particularly with IgG3 (Capra & Kunkel, 1970). However, our inhibition tests were generally performed on fresh sera and, when repeated on old samples, they did not reveal any increase in inhibition titre. None of

the sera from patients with myelomatosis displayed hyperviscosity, nor were cryoglobulins detectable in any of them.

In most diseases mentioned in the present work, circulating AgAb complexes have already been reported by other authors. Glomerulonephritis (Andres *et al.*, 1966; Michael *et al.*, 1966), hepatitis (Shulman & Barker, 1969; Almeida & Waterson, 1969; Prince & Trepo, 1971; Alpert, Isselbacher & Schur, 1971) and connective tissue disorders such as systemic lupus erythematosus (Tan *et al.*, 1966; Agnello *et al.*, 1970) and rheumatoid arthritis (Kunkel *et al.*, 1961) have now become classical examples of immune complex diseases.

For Crohn's disease we confirm the observation by Jewell & MacLennan (1973) as well as those of Doe, Booth & Brown (1973). The former reported on the presence in this disease of factors inhibiting antibody-induced cytotoxicity mediated by lymphocytes, whereas the latter have detected immune complexes by precipitation with C1q. With their C1q test, complexes were detected in 57% of the patients, whereas with our inhibition tests nearly all cases with clinical symptoms were found positive.

Idiopathic thrombocytopenia is frequently attributed to an autoimmune process (Shulman, Marder & Weinrach, 1965; Karpatkin, Garg & Siskind, 1971; Baldini, 1972). The detection of circulating AgAb complexes in this disease, our finding of reciprocal relationships between inhibition titres and the platelet count, as well as the platelet agglutinating activity of AgAb complexes (Penttinen, Vaheri & Myllylä, 1971) raise the question of a causal involvement of AgAb complexes in the pathogenesis of the thrombocytopenia.

It is known that circulating AgAb complexes can produce haemolytic effects by the 'innocent bystander' mechanism (Müller-Eberhard, 1968; Lachmann & Thompson, 1970). In our longitudinal study of Case 2 of hepatitis (Fig. 3), the haptoglobin level dropped at the time where immune complexes became detectable. A decrease in the rate of biosynthesis of haptoglobin by the diseased liver may be involved, but since the half-life of erythrocytes is decreased during hepatitis (Pitcher & Williams, 1963; Chan & Todd, 1975), the low haptoglobin concentration is probably due largely to overconsumption of this protein by excessive red cell destruction. In malignancies such as myelomatosis, leukaemia and lymphosarcoma, we have observed a high frequency of immune complexes. We do not know whether they correspond to intercurrent infections or whether the antigens they contain originate from the tumour cells. This is now being investigated especially in view of the known blocking effect of circulating AgAb complexes upon the immunological defence against tumours (Sjögren *et al.*, 1971; Baldwin, Price & Robbins, 1972). In all the cases of ankylosing spondylitis that we have examined, either agglutinating or inhibiting activity was found, suggesting that circulating immune complexes are a constant feature of this disease. These complexes presumably account for the renal lesions which Linder & Pasternak (1970) found to exist in all their cases of ankylosing spondylitis.

Urticaria is a typical symptom of reaginic allergy. However, it is known that acute hepatitis may be associated with arthritis and urticaria due to circulating AgAb complexes (Alpert *et al.*, 1971). The finding of immune complexes in our five cases of chronic urticaria makes one suspect that in this syndrome, too, they are causally involved in producing the eruptions via the anaphylactic properties of complement.

Finally there remains to be explained the presence of inhibiting factors in a high proportion (22%) of healthy individuals. Mild, inapparent, infections could of course be involved. However, since some of the sera from blood donors were collected after a meal, one should also consider enteric uptake of food antigens as a potential source of circulating AgAb complexes in healthy people. Without going into details one may cite, for instance, the observation that antibody to bovine serum albumin is detectable in the serum of 25% of normal adults (Rothberg & Farr, 1965).

Several observations reported in the present work underline the close relationships between the biosynthesis of RF and the presence of AgAb complexes in the serum. In several patients, endogenous agglutinating activity and direct inhibitory activity tended to alternate during the course of their disease. Moreover, all agglutinating sera were found to contain inhibitory factors after elimination of endogenous RF. These facts support the hypothesis that RF is produced because of stimulation by antigen–antibody complexes.

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