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Human serum inhibits the interaction between C1q or rheumatoid factor and IgG-coated latex particles. Reduction of these C1-dependent properties after complement activation *in vitro* and *in vivo*

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Summary. The interaction between human C1q or rheumatoid factor (RF) and IgG-coated latex particles was studied by means of standard aggregometer equipment. Fresh normal human serum (NHS) prevented RF from agglutinating such particles (RF-inhibiting activity) and also disagglutinated C1q-induced particle agglutinates (C1q-disagglutinating activity). Strong evidence is presented indicating that C1 was the serum factor responsible for these activities, which were lost after complement activation of serum by IgG aggregates parallel with dissociation of C1. The normal serum range for C1q disagglutinating activity is 68–100% and for RF-inhibiting activity 92–100%. Sera from the majority of patients with systemic lupus erythematosus (SLE) had reduced C1q-disagglutinating activity (78% of the patients) and reduced RF-inhibiting activity (70% of the patients) compared to sera of healthy individuals. Those patients with greatly reduced serum activities as assayed by the present techniques were in the active phase of their disorder as judged from clinical signs and reduced total haemolytic activity. The total complement in CH50 units also correlated significantly ($r=0{\cdot}73$, $P<0{\cdot}001$) with the C1q-disagglutinating activity of the patient sera. These findings together with the immunodiffusion pattern against anti-C1q and anti-C1s produced by sera from patients in the active phase of their disease suggested that circulating C1 was present in an activated and sometimes highly activated form among patients with SLE during complement consumption. Based on serial determinations it could also be excluded that functionally defective C1 was inherited or permanent among SLE patients. These techniques, which were supposed to measure functional C1 directly in serum, seemed to be sensitive and reproducible methods suitable for clinical routine in screening pathological sera and it is hoped that the methods will be useful in the treatment of patients with complement consumptive processes.

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INTRODUCTION

The first component of human complement (C1) is composed of three glycoprotein subunits, C1q, C1r and C1s, held together in a calcium-dependent complex (Naff, Pensky & Lepow, 1964; Ziccardi & Cooper, 1977). C1q binds to the antibody molecule of an immune complex and initiates the activation of the classical pathway of the humoral complement system. The change of the inactive form of C1 to the active form $\overline{C1}$ is a result of a series of intramolecular changes in the C1 molecule leading to the conversion of C1r and C1s from inactive precursor molecules to active proteases (Müller-Eberhard, 1969). Previously, it has not been feasible to quantify C1 except by activity assays

requiring specialized reagents and time-consuming techniques. In this report, evidence is presented that native C1 but not activated C1 inhibits C1q or rheumatoid factor to agglutinate IgG-coated latex particles. These findings resulted in the development of new possible methods for quantification of native C1 in normal and pathological sera.

MATERIALS AND METHODS

Assay buffer was a balanced salt solution, pH 7·2 (Na^+ 130 mmol/l, K^+ 4 mmol/l, Ca^{2+} 2 mmol/l, CH_3COO^- 30 mmol/l and Cl^- 110 mmol/l) with human albumin (AB Kabi, Stockholm, Sweden) added to a concentration of 10 g/l.

Serum samples were obtained from apparently healthy individuals or patients with SLE according to the criteria proposed by Cohen, Reynolds, Franklin, Kulka, Ropes, Schulman & Wallace (1971). Blood was drawn aseptically into plain glass tubes, allowed to clot at room temperature and centrifuged at 3000 *g* for 10 min. Serum samples were stored at −70° until used. Sera were also obtained from patients with sero-positive rheumatoid arthritis (RA). Decomplementation of serum was performed by heating at 56° for 30 min.

C1, partially purified from fresh human serum according to Cooper & Müller-Eberhard (1968), was a gift from Dr G. Stålenheim, Dept of Medical and Physiological Chemistry, University of Uppsala. The haemolytic titre of the preparation was 5×10^{13} effective molecules per ml.

C1q was prepared from fresh human serum according to the method of Yonemasu & Stroud (1972). Concentrations of C1q were determined spectrophotometrically by using the extinction coefficient of 6·8 at 280 nm (Yonemasu, Stroud, Niedermeir & Butler, 1971).

IgG of human origin was obtained from AB Kabi, Sweden. Aggregation of IgG was achieved by incubation of a 20 g/l sample at 63° for 30 min and storage at +4° for 4 weeks. The aggregates (agg IgG) treated in this way were particulate.

IgG-coated latex particles were the test reagents used for detection of conventional rheumatoid factor and obtained from Behringwerke AG, Marburg-Lahn, W. Germany. The IgG coat on the particles was of human origin. The particles were diluted 1:20 in assay buffer before being used for agglutination studies.

Rheumatoid factor (RF) was prepared from heated (56°, 30 min) serum from a patient with sero-positive rheumatoid arthritis (RA) by affinity chromatography using IgG-Sepharose. Human IgG was slightly heat-aggregated at 60° for 10 min and covalently coupled to CNBr-activated Sepharose (50 mg protein/2 ml CNBr-Sepharose, Pharmacia AB, Uppsala, Sweden). A column was poured and equilibrated with 0·2 mol/l Tris-HCl buffer pH 8, containing NaCl (0·5 mol/l). Bed dimensions were 0·9 × 5 cm. After the sample had been applied to the column, immunoadsorption was allowed to continue for 1 h. The column was washed with equilibration buffer until the absorbance at 280 nm of the effluent was negligible. The column was then eluted with 0·2 mol/l glycine–HCl at pH 2·8 containing NaCl (0·5 mol/l). The eluate, after neutralization with 0·5 mol/l NaOH and concentration, was tested by immunodiffusion against rabbit anti-whole serum, anti-IgA, -IgG, and -IgM antiserum (Behringwerke AG). The eluted material agglutinated IgG-latex particles and contained IgM at a concentration of 15 mg/l as estimated by nephelometric quantification according to the principles outlined by Lizana & Hellsing (1974).

Complement activation. Fresh normal serum was incubated with agg IgG at 37° for 60 min whereafter the particulate aggregates were removed by centrifugation in a Beckman Microfuge (Beckman Instruments Inc., Palo Alto, Ca).

Haemolytic assay. Titration of the total haemolytic complement activity of serum (CH50) was carried out by Dr K.-E. Fjellström, Dept of Internal Medicine, Uppsala according to Kabat & Mayer (1961). The normal range of CH50 in his laboratory is 40–95.

Immunochemical analyses. Immunodiffusion analyses were performed in 0·8% agarose in barbital-buffered saline pH 7·2, 0·6 × physiological ionic strength containing 2·5 mM calcium for 48 h at 4° as described by Ziccardi & Cooper (1978). C1q in serum was determined by single radial immunodiffusion in 0·8% agarose containing 10 mM EDTA. IgM, IgG, C1q and C1s in the fractions obtained after gel filtration of serum were assayed by a nephelometric technique according

to Lizana & Hellsing (1974). The antisera (Behringwerke AG) were diluted 1:50 in PBS containing polyethylene glycol (PEG 6000, Kebo, Stockholm, Sweden) at a concentration of 40 g/l. Dilutions of NHS were used as standard curves for the various proteins analysed. Anti-C1q and anti-C1s were obtained from Behringwerke AG.

Rheumatoid factor titre was determined by an acrylic test at the Department of Bacteriology, University Hospital, Uppsala.

Agglutination of IgG-coated latex particles was measured by using a standard platelet-aggregometer recording system (Payton Dual Channel Aggregation Module and Recording Equipment, Payton Associates Ltd, Scarboro, Ontario, Canada). To a glass cuvette containing a stirring bar revolving at 1000 r.p.m. was added 0·5 ml of the latex suspension. After a delay of 1 min to allow mixing and warming to 37°, 50 μl of isolated RF, heated RA-serum or C1q were added and the resulting increase in light transmission was recorded. Data evaluation of the agglutination reactions was based on the extent of agglutination. Latex particles already agglutinated by C1q were disagglutinated by the addition of 50 μl fresh serum or C1 and the extent of disagglutination was calculated. The working range of light transmission was the difference in light transmission between particles diluted in assay buffer and particles maximally agglutinated by an excess of RF.

Gel filtration was performed at 4°. Serum samples (3 ml) were applied to 90 × 1·5 cm columns of Sephadex G-200 (Pharmacia AB) or Sepharose-CL 6B (Pharmacia AB) equilibrated with assay buffer. The elution patterns were determined by optical density measurements at 280 nm.

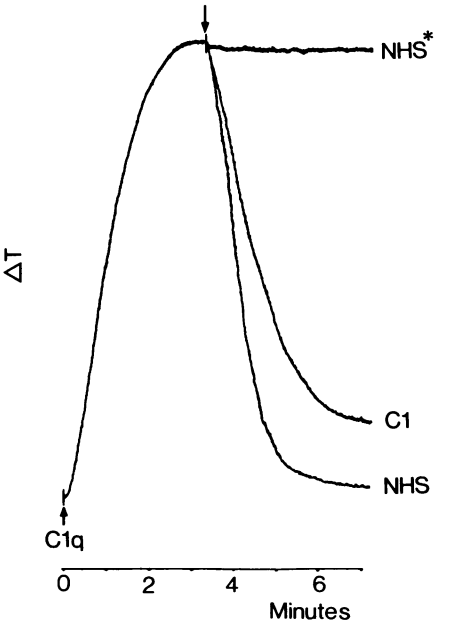

Figure 1. Agglutination of IgG coated latex particles was produced by 50 μl C1q (20 mg/l). Fifty microlitres of fresh NHS or C1 induced in contrast to heated (50°, 30 min) NHS (NHS*) disagglutination of the particle agglutinates. NHS, NHS* and C1, respectively, were added to the particles as indicated by the arrow.

RESULTS

C1-mediated disagglutination of IgG-particle agglutinates induced by C1q

IgG-coated latex particles were agglutinated by isolated C1q (20 mg/l). The addition of functionally purified C1 or fresh normal serum (NHS) to these agglutinated particles induced an immediate onset of disagglutination (Fig. 1). The disagglutination principle of NHS was heat-labile (at 56°, 30 min). Addition of various amounts of C1 to heat-treated (56°, 30 min) NHS restored its disagglutinating activity in the same dose-dependent fashion as various concentrations of fresh NHS induced disagglutination. After absorption of fresh NHS with insoluble heat-aggregated IgG (8 g/l) in the presence of EDTA, Ca^{2+} was added in amounts large enough to neutralize the chelator. Such treated serum was depleted of C1q and also of its disagglutinating property.

After separation of fresh NHS on columns of Sephadex G-200 or Sepharose-CL 6B the fractions obtained were assayed for immunoreactive C1q and C1s, respectively, and particle disagglutinating activity. The disagglutinating principle as well as the C1 subcomponents were located in the first protein peak of the G-200 serum-separation and also had identical elution patterns after separation of serum on the Sepharose column (Fig. 2). Evidently C1 was not dissociated after gel filtration since two of its subcomponents had the same k_{av}, which suggested a mol. wt of about 700,000.

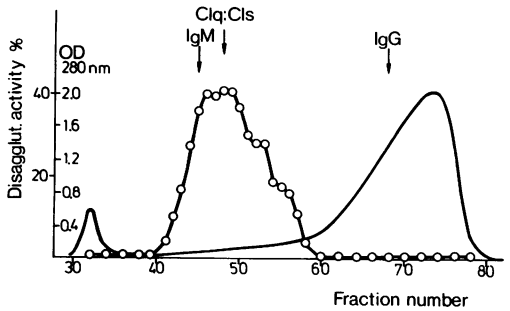

Figure 2. Fractionation pattern of serum from a healthy individual on a Sepharose-CL 6B column with optical density (OD) measurements at 280 nm (solid line). Arrows indicate maxima of the C1q, C1s, IgM and IgG peaks. All fractions of the chromatogram were tested for their ability to disagglutinate C1q-produced particle agglutinates (50 μl C1q (10 mg/l) was added to 500 μl IgG-coated latex particles diluted 1:40 in assay buffer). One hundred microlitres of each fraction were added to the agglutinates and disagglutinating activity (o) was calculated according to the formula shown in the legend to Fig 7A.

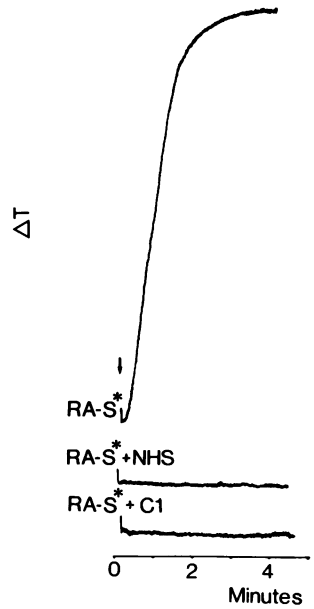

Figure 3. Fifty microlitres of heated RA-serum (RA-S*) diluted to a RF-titre 1:320 produced an immediate onset of particle agglutination. When 50 μl NHS or 50 μl C1 was added together with 50 μl RA-S* to the particles, no agglutination was obtained.

C1-mediated inhibition of RF-induced agglutination of IgG particles

Agglutination of IgG-coated latex particles was induced by the addition of purified RF of IgM type or heated sero-positive RA serum. Addition of C1 or fresh NHS to the particles prevented RF-induced particle agglutination (Fig. 3). The characteristics of the factor in NHS that inhibited RF interaction with IgG-coated particles were the same as for the serum factor that disagglutinated C1q-induced particle agglutinates.

Measurements of the activation of C1

C1 in NHS was activated by incubation at 37° for 60 min with various amounts of agg IgG (Table 1). The serum samples were subjected to CH50 determination and C1q quantification by single radial immunodiffusion in agarose containing EDTA. As shown in Table 1, complement activation as indicated by CH50 was evident after incubation of serum with 0·12 g/l of aggregated IgG while the total C1q content was reduced by about 30% with an activator dose of 2 g/l. The dissociation of C1 with activation was studied by double immunodiffusion analyses according to Ziccardi & Cooper (1978). Activated C1 produced in double immunodiffusion with anti-C1q a pattern quite different from that of precursor C1. Native macromolecular C1 gave a continuous line of precipitation with antisera to C1q and C1s. Activated C1 produced with aggregated IgG (>0·25 g/l) a spurring of the C1s precipitin line over that of macromolecular C1 (Fig. 4). The serum samples were also tested for their ability to influence the C1q and RF interactions with IgG-

Table 1. Complement activation by aggregated IgG

Amount of IgG added to NHS* (g/l)	Percentage of untreated serum		C1 precipitin line I=
	CH50	C1q	
None	100	100	+
0·12	41	100	+
0·25	13	92	−
0·5	0	88	−
1	0	75	−
2	0	70	−

* All samples were incubated at 37° for 1 h. I=, Immunodiffusion in agarose against anti-C1q and anti-C1s. +, Continuous C1 precipitin line; −, spurring of the C1s precipitin line.

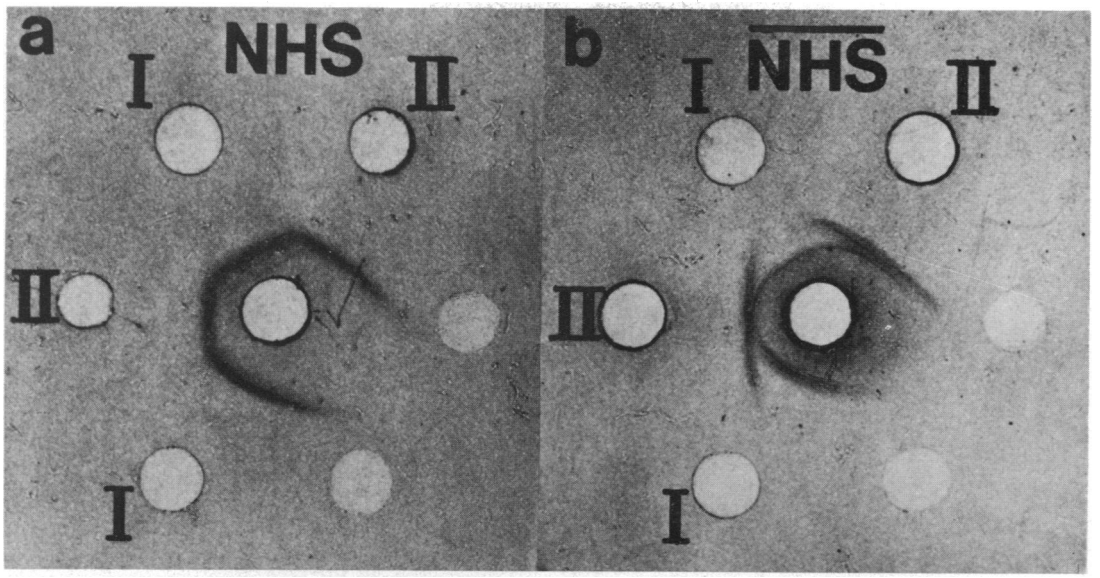

Figure 4. The effect of complement activation by aggregated IgG on the immunodiffusion pattern of C1 in human serum. Serum was allowed to diffuse in agarose containing calcium for 48 h at 4°. A continuous precipitin line against anti-C1q (I) and anti-C1s (II) was observed for untreated serum in contrast to activated serum (activated by 1 g/l of aggregated IgG at 37° for 30 min), which produced spurring of the C1s precipitin line over that of macromolecular C1.

coated particles. As depicted in Fig. 5 the ability to disagglutinate C1q-induced particle agglutinates was progressively lost as the activator dose was increased and no disagglutinating activity remained with 2 g/l of aggregated IgG. The inhibitory effect of RF mediated agglutination was also progressively lost with amounts of agg IgG exceeding 0·1 g/l and completely lost with larger activator doses (agg IgG ≥ 1 g/l) (Fig. 6).

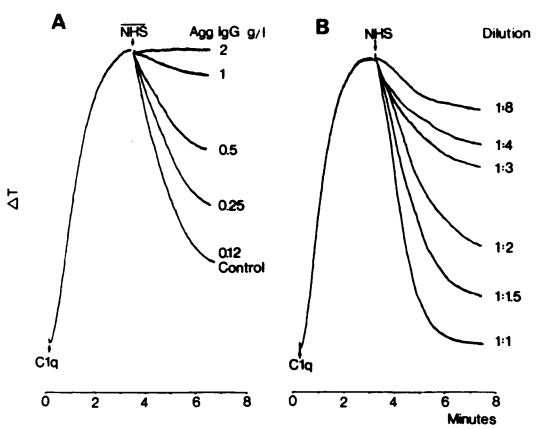

Figure 5. IgG-coated particles were agglutinated by 50 μl C1q (20 mg/l). (A) Fresh NHS was activated ($\overline{\text{NHS}}$) by various amounts of aggregated IgG at 37° for 60 min and 50 μl of each $\overline{\text{NHS}}$ sample was added to the particle agglutinates as indicated by the arrow. The disagglutinating activity of $\overline{\text{NHS}}$ was progressively lost with larger amounts of aggregated IgG. (B) fifty microlitres of various dilutions of fresh $\overline{\text{NHS}}$ were tested for their disagglutinating activities.

The C1q-disagglutinating and RF-inhibiting activities of normal and SLE sera

The ability of NHS to disagglutinate C1q-induced particle agglutinates is named C1q-disagglutinating activity. The procedures to test this activity include, as a first step, agglutination of IgG particles by the addition of 50 μl C1q (20 mg/l). After 3 min of incubation (when agglutination was completed) 50 μl of undiluted serum were added to the incubate and the extent of disagglutination produced by serum was read. The activity was then calculated according to the formula given in Fig. 7A. The C1q-disagglutinating activity of healthy controls ($n=28$) had a mean of 90% (68–100% 2 SD). The disagglutinating activity of SLE sera varied between 0 and 100% and correlated well with the CH50 titre ($r=0{\cdot}73$, $P<0{\cdot}001$) as shown in Fig. 8. The test as described only measures up to 100% disagglutination. In order to discriminate the disagglutinating property of normal and pathological serum specimens with high levels of this property such specimens were diluted 1:2 or 1:4. In those normal subjects and in six

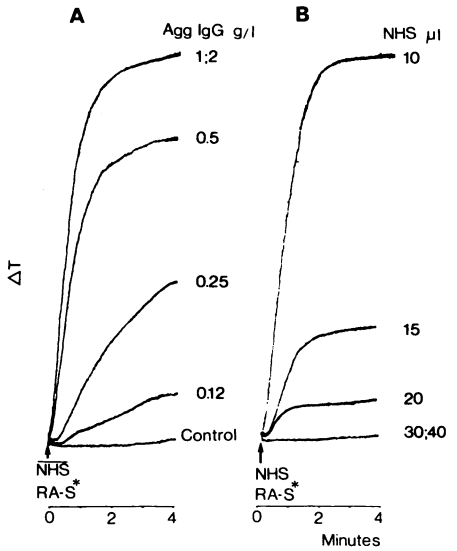

Figure 6. Fifty microlitres of a heated RA-serum (RA-S*) diluted to a RF titre 1:320 mediated a particle agglutination. (A) Fifty microlitres of fresh NHS which was activated (NHS) by various amounts of aggregated IgG at 37° for 60 min was added together with 50 µl RA-S* to particles. The control serum sample (50 µl) inhibited completely the agglutination of particles by RA-S*, while the serum inhibitory activity was gradually lost with larger activator doses. (B) Fifty microlitres of the RA-S* was added together with various amounts of fresh NHS to the particles.

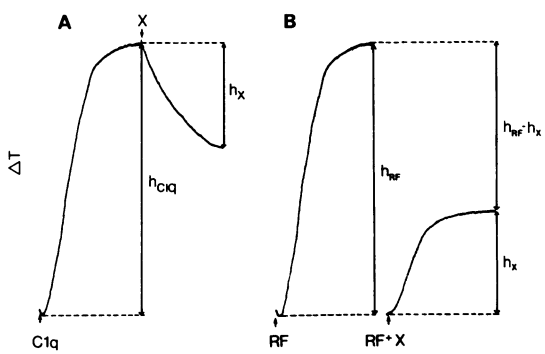

Figure 7. (A) Estimation of the C1q-disagglutinating activity of serum. The extent of particle agglutination produced by C1q (h_{C1q}) and the disagglutination (h_X) produced by a sample X were determined. The disagglutinating activity for sample X was calculated according to the formula: $(h_x/h_{C1q}) \times 100$. (B) Estimation of the RF-inhibiting activity of serum. The agglutination pattern produced by heated RA-serum is illustrated and the extent of agglutination (h_{RF}) is calculated. The extent of agglutination produced in the presence of a sample X is called h_X. The inhibition of agglutination mediated by sample X is calculated according to the formula: $[(h_{RF}-h_X)/h_{RF}] \times 100$.

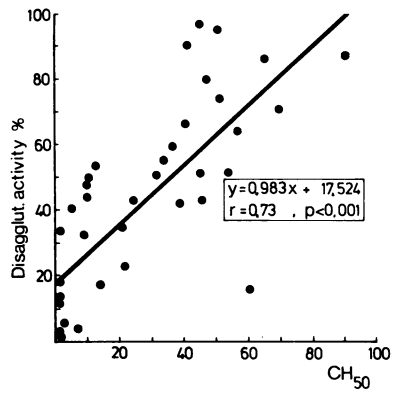

Figure 8. C1q-disagglutinating activity versus CH50-titre of sera from thirty-six patients with SLE. The 2 SD range of C1q-disagglutinating activity for healthy control sera is 68–100%.

patients with high CH50 titres, however, no correlation was obtained at any serum concentration between the CH50 titre and values obtained in the disagglutination test.

The RF-inhibiting activity of sera was tested by simultaneous addition of 50 µl of pooled RA-sera (diluted to a RF titre of 1:320) and 20 µl of the serum sample. The inhibition of agglutination was calculated according to the formula in Fig. 7B. The RF-inhibiting activity of control sera ($n=28$) was on average 97% (92–100% 2 SD). Twenty-three SLE sera were available for determination of RF-inhibiting activity, which ranged between 0 and 96%.

A linear relationship between the C1q-disagglutinating and RF-inhibiting activties of SLE sera was found but only within the subnormal ranges of the activities (Fig. 9). Those SLE patients, whose sera had pronounced reduction of C1q-inhibiting (<20%) or RF-inhibiting activities (<60%) were all clinically in active phases of their disorder. Addition of NHS at a final concentration of 30% or purified C1 to these sera normalized both activities indicating a deficiency of native C1.

These pathological sera were also investigated by immunodiffusion against anti-C1q and anti-C1s and the precipitation patterns were the same as those found for NHS activated by agg IgG (Fig. 4), suggesting dissociation of C1. In contrast, all healthy control sera exhibited continuous C1 precipitin lines. Serial determinations of three SLE patients over a period of about 1 year showed that both C1q-disagglutinating and RF-inhibiting activities increased and in one case

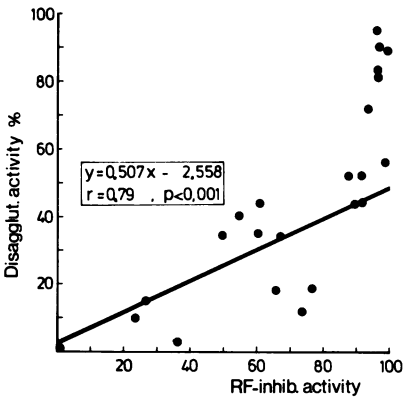

Figure 9. C1q-disagglutinating activity versus RF-inhibiting activity of sera from twenty-three patients with SLE. The 2 SD normal range for RF-inhibiting activity is 92–100%. The relationship between the two variables is linear within the subnormal ranges of the two activities.

normalized during clinical improvement, supporting the idea that the presence of dissociated and functionally defective C1 in SLE sera is not permanent but varies with the activity of the disorder.

A recently reported test for measuring circulating immune complexes (Lurhuma, Cambiaso, Masson & Heremans, 1976) is based on the principle that soluble immune complexes have the property of competing for C1q and RF and inhibiting IgG coated latex agglutination. Therefore, the possible influence of soluble IgG aggregates (produced as previously described but without storage) on the present agglutination reactions was investigated. Such aggregates at various concentrations (0·1–2 g/l) in buffer or in NHS* mediated no disagglutination of C1q-induced particle agglutinates. A slight inhibition of the RF-mediated particle agglutination was observed but only at the highest aggregate concentration. These results show that circulating immune complexes in practice have no direct influence on the measurements made in the present systems. The effect on the tests of intrinsic RF in those sera which possess it in appreciable quantities is a more complex matter. As reported in detail elsewhere (Hällgren, 1979), such sera also contain dissociated C1 as shown by the immunodiffusion technique of Ziccardi & Cooper (1978) and at least partly for that reason have low disagglutinating levels.

DISCUSSION

Intact C1 can be measured by time-consuming haemolytic techniques (Borsos & Rapp, 1963) and activated C1 can be quantified by more complex haemolytic techniques which involve specialized reagents and multiple steps (Colten, Borsos & Rapp, 1969). Laurell *et al.* (Laurell, Mårtensson & Sjöholm, 1976, 1977; Johnsson, Kamme, Laurell & Nilsson, 1977) demonstrated by crossed immunoelectrophoresis that some pathological sera contain high amounts of C1r and C1s proteins not associated with C1q. Recently Ziccardi & Cooper (1978) presented an alternative approach to quantify C1 activation in serum based on the finding that C1 dissociates during the activation process. They found that activated C1 gave a different precipitation pattern than native C1 with antisera to C1q, C1r and C1s and that these alterations could be quantified directly by single radial immunodiffusion techniques. Such techniques to quantify C1 activation directly in serum are simpler to perform compared with previous techniques, but involve specific antisera of which at present only anti-C1s and anti-C1q are commercially available.

In this investigation, two new approaches to measure C1 activation in serum are presented based on previous observations that native C1 interferes with the interactions of C1q or RF with IgG bound to latex particles (Hällgren, 1979; Hällgren, Stålenheim & Venge, 1979). C1q and RF both mediate agglutination of such particles and the kinetics of agglutination can be measured by a standard platelet-aggregometer system. As previously reported and further illustrated in this study, however, IgG particle agglutinates produced by C1q are disagglutinated by native C1 and the particle agglutination mediated by RF is inhibited by non-activated C1. In contrast, activated serum obtained after incubation with complement activators such as aggregated IgG exhibited no or reduced interference with the particle agglutination mediated by C1q or RF. The lowest activator dose examined (agg IgG 0·12 g/l) gave no dissociation of C1 as examined in double immunodiffusion techniques with anti-C1s and anti-C1q, but interfered with the particle agglutination mediated by C1q or RF. With higher activator doses the C1q disagglutinating and the RF-inhibiting activities of serum were gradually lost in parallel to dilutions of the fresh untreated serum. At higher activator doses (agg IgG > 0·25 g/l) the Ouchterlony pattern suggested C1 dissociation. After heating serum at 56° for 30 min, which destroys the heat-labile C1 molecule, the interference of serum on the C1q and RF interactions with IgG-coated particles was completely lost but restored by the addition of native C1.

To define further the basis of this serum property fresh NHS was fractionated on Sephadex G-200 or Sepharose-CL 6B columns. The disagglutinating or RF-inhibiting curves were exactly parallel to the C1q or C1s concentration curves. The same elution pattern for C1q and C1s indicated that these proteins were complexed. The calculated molecular weight of that complex was 700,000 suggesting that C1q and C1s were parts of the native C1 complex (Ziccardi & Cooper, 1977). Present data together with previous observations strongly suggest that intact C1 is the fresh serum factor which produces an inhibitory action on C1q and RF binding to IgG and that this C1 action is lost after activation of C1. The most probable basis of the C1 activity is the competition between native C1, with a higher affinity for IgG coated particles, and C1q or RF.

The present attempts to quantify the amounts of native C1 in serum were applied to sera from healthy controls and patients with SLE. All normal sera tested inhibited RF to induce particle agglutination and produced a very similar disagglutinating action on C1q-induced particle agglutinates. In contrast, many of the SLE sera had a completely or largely reduced C1q disagglutinating or RF-inhibiting activities. A significant correlation was obtained between the CH50 titre of the patients' sera and their C1q-disagglutinating activity, indicating that circulating activated C1 is present in the sera of patients with complement consumptive diseases such as SLE. Support to this idea was given by the immunodiffusion patterns produced by sera from patients with active SLE against anti-C1q and anti-C1s which were quite similar to those found in normal sera activated by immune aggregates.

Based on the relationship between C1q-disagglutinating and RF-inhibiting activities in SLE sera and on experimental data, these assays of human C1 evidently measure the same C1 activity but the methods have different sensitivities within the normal serum C1 range. The mechanisms by which C1 is activated in SLE patients are unknown, but presumably mediated by circulating antigen–antibody complexes (Nydegger, Lambert, Gerber & Miescher, 1974; Hällgren & Wide, 1976). A permanent functional deficiency of C1 in SLE can be excluded since serial determinations on such patients revealed that both the C1q-disagglutinating and RF-inhibiting activities varied with clinical conditions and CH50 titre.

One limitation of the present techniques is that they cannot discriminate between reduced serum levels of native C1 caused by reduced C1 production as opposed to C1 activation. This limitation can be partly overcome by quantification of serum C1q. It remains to be determined whether the techniques presented here, which are easily performed and have a high capacity may be a supplementary aid in the management of patients with complement consumptive processes. Investigations in progress are also aimed at answering what the relationship in normal and pathological serum specimens is between the haemolytic C1 activity and the C1-dependent activity as described in this report.

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