Circulating Immune Complexes in the Serum in Systemic Lupus Erythematosus and in Carriers of Hepatitis B Antigen

QUANTITATION BY BINDING TO RADIOLABELED C1q

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ABSTRACT A sensitive and reproducible procedure for the detection of soluble immune complexes in sera from patients with various immunopathological disorders is reported. Radiolabeled C1q is reacted with sera containing immune complexes. Separation of free from complex bound [^{125}I]C1q is achieved by selective precipitation with polyethylene glycol (PEG). The method is based on both the large molecular size and the C1q-binding property characterizing immune complexes. The minimal amount of aggregated immunoglobulins thus detected is about 10 μg and that of soluble human IgG-anti-IgG complexes is about 3 μg of complexed antibody. Some immune complexes formed in large antigen excess (Ag_2Ab) can still be detected by this radiolabeled C1q binding assay. The specificity of the radiolabeled C1q binding test was documented by the inability of antigen-F(ab')$_2$ antibody complexes to lead to a precipitation of [^{125}I]C1q in PEG.

In a second step, this radiolabeled C1q binding assay was applied to an experimental model of immune complex disease and was shown to be efficient for the detection of in vivo formed immune complexes.

Finally, the technique could be applied to the study of sera from patients with systemic lupus erythematosus (SLE) or to carriers of the hepatitis B antigen (HB-Ag). Significantly increased [^{125}I]-C1q binding values were observed in 52 sera from SLE patients when compared to values obtained with healthy blood donors ($P < 0.001$). Particularly high values were seen in active disease, a finding which was confirmed by follow-up studies performed with four SLE patients.

No increased [^{125}I]C1q binding was seen in 18 healthy carriers of the HB-Ag; whereas, sera from carriers with hepatitis appear to precipitate increased [^{125}I]C1q percentages: 7/24 cases with acute transient and 4/7 cases with chronic persistent hepatitis were found to increasingly bind [^{125}I]C1q. The results were also used for a correlative study of [^{125}I]C1q binding to IgG levels in the sera but increased [^{125}I]C1q binding could not be attributed to high serum IgG levels which are likely to account for gammaglobulin aggregates.

These examples suggest the utility of the radiolabeled C1q binding assay for the evaluation of immune complex diseases in human pathology.

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INTRODUCTION

A variety of immunopathological disorders appear secondary to the circulation of immune complexes or to their deposition into tissues. It was clearly demonstrated that soluble immune complexes, circulating in antigen (Ag)[1] excess during chronic or acute experimental serum sickness, become localized in vascular walls and

[1] *Abbreviations used in this paper:* Ab, antibody; agg HGG, aggregated human IgG; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; HB-Ag, hepatitis B antigen; LDV, lactic dehydrogenase virus; NDNA, native DNA; NHS, normal human serum; NRS, normal rabbit serum; PBS, phosphate buffered saline; PEG, polyethylene glycol; ppt, precipitated; SDNA, single-stranded DNA; SLE, systemic lupus erythematosus; VBS, Veronal-buffered solution.

particularly in renal glomerular capillaries (1). These experimental models have contributed to the understanding of immune complex disease in man and there exists considerable evidence that circulating immune complexes play a pathogenic role in systemic lupus erythematosus (SLE) (2) and in some cases of bacterial (3), viral (4), or parasitic (5) infections.

Several approaches have been used to demonstrate immune complexes in serum or tissues. The existence of tissue-bound immune complexes has been suggested mainly by immunohistological techniques (6, 7), showing the presence of immunoglobulins, complement components, and sometimes antigen in the lesions. More direct techniques led to the demonstration in serum samples of immune complexes involving a previously identified antigen. For instance, detection of hepatitis B antigen (HB-Ag)-antibody (Ab) complexes could be done by using electronmicroscopy (8) or detection of lactic dehydrogenase virus (LDV)-antibody complexes was achieved in mice by the demonstration of a decreased infectivity of LDV in serum after precipitation of the circulating immunoglobulins (9). Detection of circulating immune complexes can also be accomplished by procedures which take advantage of the great molecular weight of immune complexes such as chromatography (10) and ultracentrifugation in sucrose density gradients (11). Further procedures are based on the steric modifications of the Fc fragments of immunoglobulins occurring during the formation of immune complexes. Firstly, the particular reactivity of immune complexes with rheumatoid factors was used for their detection in serum or joint fluid of various patients (12). Secondly, the complement-fixing properties of immune complexes has been used for their demonstration. Several authors used as a test the evaluation of anticomplementary activity of the whole serum.

The C1q component of complement contains the site through which the first component combines with gammaglobulin (13) and it is precipitated by soluble immunoglobulin aggregates (14). This property was applied successfully to a practical test in which agar gel precipitation was used (15). This gel diffusion method has been widely applied to the detection of soluble immune complexes in sera from patients with SLE, rheumatoid arthritis (16), dermatitis herpetiformis (17), leprosy (18), and polyarteritis nodosa (19) and in joint fluids from patients with rheumatoid arthritis (16).

The method which will be described in this paper is based on both the large molecular size and the C1q-binding properties characterizing immune complexes. It is known that the immune precipitation of antigen-antibody complexes is increased in polyethylene glycol (PEG) (20). This compound can be used to fractionate plasma proteins according to the size of the molecules (21). In previous studies, PEG was used at low concentrations in order to precipitate soluble antigen-antibody complexes in conditions at which free antigen or free antibody would be soluble (22).

Similarly, PEG proved to be a useful agent in separating free [^{125}I]C1q from [^{125}I]C1q bound to immune complexes in a fluid phase system. Radiolabeled C1q was mixed with serum containing, or not containing, immune complexes. PEG was then added at a concentration which would result in precipitation of [^{125}I]C1q bound to macromolecular aggregates while unbound [^{125}I]C1q would remain quite soluble.

The method was first developed using in vitro formed immune complexes and aggregated immunoglobulins in order to define the binding properties of radiolabeled C1q and the effects of PEG.

In a second step, this radiolabeled C1q binding assay was applied to an experimental model of immune complex disease and was shown to be efficient for the detection of in vivo formed immune complexes. Finally, the method was applied to the investigation of patients with diseases known for their association with circulating immune complexes: SLE patients and HB-Ag carriers.

The results suggest that the radiolabeled C1q binding assay can be used for detection of circulating immune complexes, and therefore can be a useful adjunct in the diagnosis and follow-up of immune complex disease.

METHODS

Preparation of C1q labeled with ^{125}I or ^{131}I. The C1q component of complement was prepared from fresh human serum by precipitation with DNA and gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden) as described by Agnello, Wichester, and Kunkel (15). The purity of C1q was demonstrated by immunoelectrophoresis, by using anti-human total serum and anti-C1q serum.

Radio-iodination was performed in lactoperoxidase-catalyzed reactions in phosphate (0.01 M)-buffered saline (0.15 M) (PBS) as described by Heusser, Boesman, Nordin, and Isliker (23). Lactoperoxidase was obtained from Sigma Chemical Co., St. Louis, Mo., and the Na^{125}I and Na^{131}I from the Radiochemical Centre, Amersham, England. The labeled C1q was then exhaustively dialyzed against PBS to remove non-protein-bound isotopes and divided into samples of 0.1 ml containing 40–60 μg C1q (sp act 0.1–0.5 μCi/μg C1q). For each test series, one sample was thawed, diluted in isotonic Veronal-buffered saline (VBS) (24) and centrifuged for 30 min at 7,000 g and 4°C in a Sorvall RC-2B centrifuge (Ivan Sorvall, Inc., Newton, Conn.) to remove aggregated protein. The supernate was then used for the C1q binding test described below.

Preparation of immune reactants. Human IgG was prepared according to Kistler and Nitschmann (25) and was supplied by the Central Laboratory of the Swiss Red Cross (Berne, Switzerland). It contained IgG, IgA, and IgM in a ratio of 600:7:1. Heat aggregation was performed for 20 min at 60°C and the aggregates (agg HGG) used

freshly. Bovine serum albumin (BSA) was obtained as a lyophilized preparation from Behringwerke, Marburg/Lahn, Germany. Both the human IgG and BSA were trace labeled with ^{125}I by means of chloramine T coupling as described by McConahey and Dixon (26).

Human IgG and BSA were used as antigens for immunization of rabbits in complete Freund's adjuvant (CFA). Anti-human IgG and anti-BSA antibodies were measured by classical precipitation techniques (27). The pools of antisera used contained 6–7 mg/ml of anti-BSA and 2–3 mg/ml of anti-IgG antibody, respectively. IgG-anti-IgG and BSA-anti-BSA complexes were formed by reacting increasing concentrations of the respective antigen with constant amounts of heat-inactivated antiserum under the same conditions as described for the [^{125}I]C1q binding test.

F(ab')$_2$ fragments of anti-BSA antibody were produced as follows: IgG of immunized rabbits were obtained by chromatography on DEAE-cellulose. Digestion of the eluted IgG was performed with pepsin (Worthington Biochemical Corp., Freehold, N. J.) for 18 h at 37°C, pH 4.0 (28). The preparation was then dialyzed against PBS and eluted on a Sephadex G-200 column (60 × 1.8 cm). The digested anti-BSA IgG reacted with BSA by immunodiffusion but not with anti-rabbit Fc antibody. Reduction and alkylation of the patients' sera or of agg HGG was achieved by incubation with mercaptoethanol as described by Agnello et al. (15).

Soluble complexes were prepared as follows: 50 times the amount of antigen (IgG) corresponding to the zone of equivalence was reacted with anti-IgG antiserum for 3 days at 4°C. Thereafter, the complexes were centrifuged at 2,000 *g* for 45 min at 4°C and the supernates were used for the experiments.

Heat inactivation of normal human serum (NHS) or normal rabbit serum (NRS) was performed for 30 min at 56°C. Gel filtration of NHS or sera from patients with SLE on Sephadex G-200 was done by downward flow on a 1.8 × 70-cm column at a rate of 5–6 ml/h using PBS for elution. The various fractions were pooled with respect to the different elution peaks and concentrated to the volume of the starting material.

Induction of acute serum sickness in the rabbit. In vivo formation of soluble immune complexes was achieved by producing acute serum sickness in four random-bred rabbits. By slightly modifying the procedure described by Dixon et al. (1, 29), the rabbits were given 20 mg of BSA in CFA by intramuscular injection 3 days before injecting them intravenously 200 mg/kg of a trace-labeled BSA solution. The rabbits were bled daily in the ears and the immune elimination of the antigen was followed by comparing the radioactivity in the blood with that of the injected material. Simultaneously, the serum samples were assessed for [^{131}I]C1q binding and for the presence of antibody-bound BSA by means of the Farr technique (30).

Immunological techniques. Anti-DNA antibodies were assessed according to Minden and Farr (31) and to Wold, Young, Tan, and Farr (32). DNA was labeled with tritium. It was prepared by cultivation of human fibroblast cells in presence of tritiated thymidine. The tritiated DNA ([^{3}H]-DNA) was extracted and purified according to Marmur's technique (33). Single-stranded DNA (SDNA) was prepared by heating native DNA (NDNA) at 100°C for 10 min and transferring immediately to an ice bath. The results of the DNA binding test are expressed as a percent of 0.2 μg [^{3}H]DNA which was bound to antibody and therefore precipitable by 50% saturated ammonium salt.

Solubilities of ^{125}I trace-labeled BSA, human IgG, agg HGG, and of [^{125}I]C1q were assessed with different concentrations of PEG (mol wt 6,000, DAB-7, obtained from Siegfried, Zofingen, Switzerland) dissolved in VBS at various concentrations between 0.5 and 20% (wt/vol). The radioactive proteins were suspended in NHS and VBS. 0.4 ml of these preparations were mixed with 3 ml of PEG at various concentrations. Final incubations were for 60 min at 25°C and for 3 h at 4°C. After centrifugation at 1,000 *g* for 20 min the supernates were discarded and the percentage of precipitated proteins was calculated from the results of radioactivity counting.

Complement hemolytic assays were performed by using a continuous flow system (34). They were used for estimation of plasma complement activity in patients and for complement fixation analysis. Agg HGG were tested for complement fixing activity by incubation of various concentrations of agg HGG with 50% fresh serum as a source of complement for 90 min at 37°C and 20 h at 2–4°C.

IgG, IgA, and IgM quantitations were carried out by using the single radial immunodiffusion method (35).

Radiolabeled C1q binding test. For the experimental part of the assays, 0.2 ml of various concentrations of immune reactants were added to 0.2 ml of heat-inactivated NHS, which was diluted to a final concentration of 1/20. For the application to immune complex detection, 0.2 ml nondiluted patient's serum was mixed with 0.2 ml VBS. Thereafter, the test steps were identical for both cases, i.e., 50 μl [^{125}I]C1q containing 1 μg/ml of C1q was added to each tube. All tests were done in duplicate. The mixtures were incubated for 60 min at 25°C and for 60 min at 4°C. In a second step, 3 ml of PEG was added to a final concentration of 2.5%. Incubation took place at 4°C for 2 h. The tubes were then centrifuged at 1,000 *g* for 20 min, the supernates were discarded, and radioactivity was measured in the precipitates. Additional washing of the precipitates in PEG did not lead to a significant decrease of nonspecific precipitation. Results were expressed as percent [^{125}I]C1q precipitated (ppt). They were calculated on the basis of protein-bound radioactivity (precipitable by 10% trichloroacetic acid) which was assayed in each test run. In some preliminary experiments with preformed immune complexes, the results were corrected for nonspecific precipitation of [^{125}I]C1q by the method of Farr (30). However, this correction was not applied to patient studies in order to allow for a better statistical evaluation of the results as compared with the control sera.

52 sera were obtained from 22 patients suffering from SLE. Blood was allowed to clot at room temperature for 60 min. Serum was separated by centrifugation at 4°C. It was used fresh or after storage at −70°C. Samples for estimation of hemolytic complement activity were drawn on EDTA at a wt/vol ratio of 2.5 mg/ml blood. SLE disease activity was judged on the following criteria: active nephritis (proteinuria, hematuria), creatinine clearance, blood urea nitrogen, hemolytic complement activity, and DNA binding activity. Serial bleedings were performed with four of the 22 patients.

54 sera were also obtained from 50 carriers of the HB-Ag. HB-Ag was demonstrated by counterimmunoelectrophoresis (36). Circulating HB-Ag was quantitated by using a radiolabeled Fab fragment from purified anti-HB-Ag antibody in a coprecipitation system with PEG (37). The results are expressed in percent of [^{125}I]Fab anti-HB-Ag precipitated and are corrected for nonspecific precipitation by Farr's formula (31). The sera were grouped into three

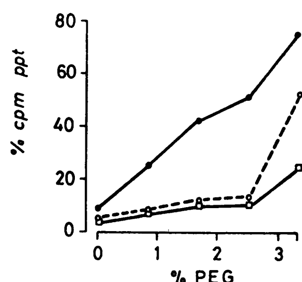

FIGURE 1 Solubilities of trace-labeled agg HGG (□——□), [^{125}I]C1q (O - - - O), and [^{125}I]C1q mixed with agg HGG (●——●) at various PEG concentrations. % PEG indicates final concentrations. Whereas the single constituents are quite soluble at 2.5% PEG, one can see that the [^{125}I]-C1q mixed with agg HGG is in part precipitable in presence of 1–2.5% PEG.

classes, according to the clinical state of the carriers. 18 carriers were symptomless. Neither their general physical and laboratory examinations nor their past history revealed clinical or biochemical evidence for present or past liver disease. Eight of these carriers did not have hepatitis as documented by histologic examination of liver biopsies. The criteria recommended by Reinicke et al. were applied to the description of all subjects (38).

24 carriers of the HB-Ag suffered from acute transient hepatitis and seven from chronic persistent hepatitis. Diagnosis of infectious hepatitis was established according to the criteria of Sherlock (39) and Goldberg (40). The subdivision into "acute transient" and "chronic persistent" disease was based on the evolution of the case history and biochemical parameters. Furthermore, 23 cases were documented by liver biopsy (Tables III and IV).

The type of therapy in SLE patients and HB-Ag carriers was not selected in a prospective or random manner. The [^{125}I]C1q binding activity is only intended to be compared with the actual state of the disease.

The statistical differences were calculated by a paired t test. Estimation of population regression curves from single data was done by the method of least squares (41).

RESULTS

Separation of free [^{125}I]C1q from [^{125}I]C1q bound to agg HGG or to immune complexes. To separate free [^{125}I]C1q from [^{125}I]C1q bound to macromolecular complexes, procedures based on the principle of solvent exclusion according to the molecular weight were investigated. It was found that PEG could be used as a fractionating medium for this purpose. The solubility of [^{125}I]C1q in increasing concentrations of PEG was studied in presence of heat-inactivated NHS either with or without addition of agg HGG (2 mg/ml) suspended in NHS. The addition of NHS to these preparations was done in order to maintain the final protein concentration approximately constant. For convenience, a single centrifugation rate of 1,000 g for 20 min was chosen. The results are shown in Fig. 1. While agg HGG and [^{125}I]C1q alone remain quite soluble at final PEG concentrations of up to 2.5%, one can see that up to 50% of [^{125}I]C1q-agg HGG complexes are precipitated by even lower PEG concentrations. Therefore, a final PEG concentration of 2.5% was chosen for the ensuing experiments. In the conditions used, only 50 ng of [^{125}I]C1q was added to 400 μg of agg HGG. Only unspecific precipitation of [^{125}I]C1q-agg HGG complexes is detected if VBS is substituted for PEG in this experiment.

Various concentrations of agg HGG were reacted with [^{125}I]C1q in presence of heat-inactivated NHS before the addition of 2.5% PEG. With increasing concentrations of agg HGG, an increased percentage of [^{125}I]C1q is precipitated, reaching complete precipitation for 400 μg agg HGG. The preparation of agg HGG contained 48% IgG molecules in aggregated form as found by gel filtration on Sephadex G-200. Therefore, since 20 μg agg HGG still led to a significant precipitation of [^{125}I]C1q, one should consider that the minimal amount of agg HGG which can be detected by [^{125}I]C1q precipitation is around 10 μg. The addition to [^{125}I]C1q of unheated immunoglobulins, which are likely to contain minimal amounts of aggregates, also leads to some precipitation of [^{125}I]C1q. However, the quantities of native IgG needed for 50% precipitation of [^{125}I]C1q are 10–20 times higher than those needed for agg HGG. The effect of PEG could be demonstrated when agg

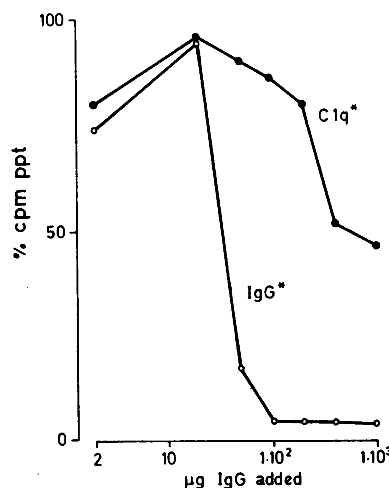

FIGURE 2 Radiolabeled C1q binding test in presence of in vitro formed immune complexes. Increasing amounts of human IgG were added to a constant amount of anti-IgG-antibody. The upper curve (●——●) represents the values of [^{125}I]C1q precipitation in the test. The lower curve (O——O) represents the percentage of IgG ppt by the antibody in similar conditions but in the absence of [^{125}I]-C1q and of PEG. A significant [^{125}I]C1q precipitation is still observed in far antigen excess. IgG ppt is corrected for nonspecific precipitation according to Farr's formula. Nonspecific [^{125}I]C1q ppt by NHS was 12%.

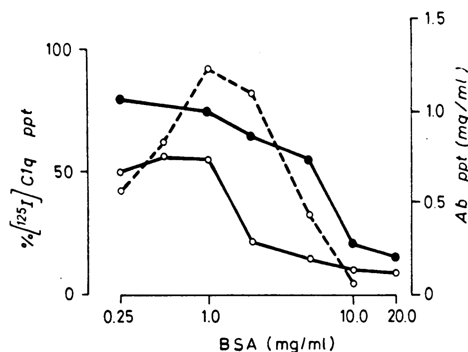

FIGURE 3 Radiolabeled C1q test in the presence of BSA-anti-BSA complexes. Increasing amounts of BSA added to a constant amount of antibody. The amount of antibody ppt was measured in the absence of PEG (○- - -○). The percentage of [125I]C1q ppt by addition of the immune complexes was studied either in presence (●——●) or in absence (○——○) of PEG. The enhancing effect of PEG on the precipitation of bound [125I]C1q is clearly shown in the equivalence zone and in slight antigenic excess.

HGG were incubated with [125I]C1q in the absence of PEG. No significant [125I]C1q precipitation occurred even at high concentrations of agg HGG.

The precipitation of [125I]C1q in presence of immune complexes and 2.5% PEG was first studied using an IgG-anti-IgG system. Increasing amounts of human IgG were reacted with a constant amount (60 μg) of rabbit anti-human IgG antibodies and 50 ng of [125I]-C1q. In a parallel experiment, the precipitation of the antigen was measured classically, by using trace amounts of [125I]IgG in the absence of [125I]C1q and PEG. As shown in Fig. 2, 95% of [125I]C1q was precipitated in Ag/Ab ratios at which a maximal antigen precipitation was observed and corresponding, in fact, to the zone of equivalence. Furthermore, 46% of [125I]-C1q probably bound to macromolecular complexes was still precipitated by 2.5% PEG at Ag/Ab ratios reaching 40–50 times Ag excess as compared to the point of maximal antigen precipitation.

When the [125I]C1q precipitation was studied in a similar experiment performed without any addition of PEG, 57% of [125I]C1q was precipitated at Ag/Ab ratios corresponding to the maximal antigen precipitation, but this percentage decreased rapidly in antigen excess. No significant precipitation of [125I]C1q was observed in 2.5 times antigen excess. The addition of PEG modifies the solubility of both complex-bound [125I]C1q and of soluble IgG-anti-IgG complexes. Indeed, when the precipitation of [125I]IgG was measured in this antigen-antibody system in the presence of 2.5% PEG, it was found that 19% of [125I]IgG was still precipitated at 50 times antigenic excess as compared to 1.2% in the absence of PEG.

In another experiment, soluble IgG-anti-IgG complexes were formed in 50 times antigenic excess in presence of 240 μg of antibody. After separation of any remaining insoluble complexes, such soluble complexes were diluted serially in heat-inactivated NRS, incubated with [125I]C1q then with 2.5% PEG. It was seen that immune complexes containing a maximal amount of approximately 3 μg of antibody are still modifying the solubility of [125I]C1q in 2.5% PEG. This value is slightly overestimated since some of the antibody has been removed in the form of insoluble complexes before setting up the C1q precipitation.

The binding of [125I]C1q to immune complexes was also studied by incubating increasing concentrations of BSA with constant amounts of rabbit anti-BSA antibodies in presence of 50 ng of [125I]C1q and with further additions of PEG or VBS. Simultaneously, the precipitation curve was studied for this particular BSA-anti-BSA system by measuring the amount of BSA and antibody precipitated. Fig. 3 represents the results obtained. The [125I]C1q precipitation appears largely to parallel the precipitation curve. However, in presence of soluble complexes formed in antigenic excess, a significant [125I]C1q precipitation was still found after addition of PEG; whereas, very little [125I]C1q precipitation was observed at these antigen concentrations in the absence of PEG.

Comparative studies: specificity and reproducibility. Comparative investigations were undertaken in order to test the sensitivity of the [125I]C1q precipitation assay. Firstly, this sensitivity was compared to that of the C1q agar gel precipitation assay. Decreasing concentrations of agg HGG were assessed against a constant amount of C1q according to the procedure previously described (15). Concentrations >1 mg/ml of agg HGG resulted in strong precipitation lines and a faint line was still observed for 500 μg/ml of agg HGG. In

TABLE I

*Specific [125I]C1q Precipitation by Equivalence-Formed BSA-Anti-BSA Complexes: Comparison of Complexes Formed with Intact Antibody or with $F(ab')_2$ Fragment Only**

	Antibody concn (*mg Ab/ml*)			
	1.0	0.5	0.25	0.12
IgG antibody	78‡	30.5	7	0
$F(ab')_2$ *antibody*	3	0	1	0

* Test conditions: BSA-anti-BSA complexes were formed at equivalence. The reaction product was serially diluted in NRS and used for [125I]C1q precipitation assays in presence of PEG at 2.5%.

‡ Percent [125I]C1q ppt, corrected for nonspecific precipitation in presence of NRS.

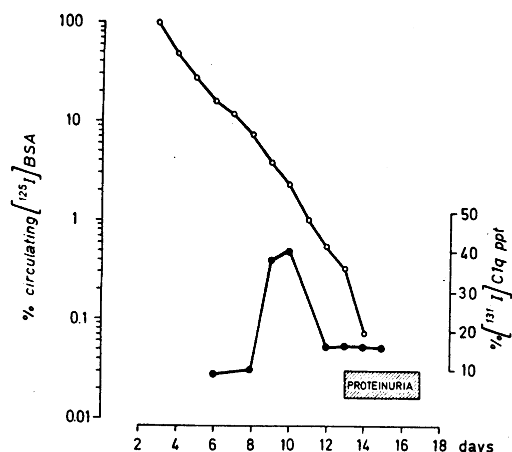

FIGURE 4 Radiolabeled C1q test in experimental serum sickness. Elimination of the BSA antigen (○——○) follows a classical curve with 99.9% of BSA eliminated 13–15 days after immunization with BSA. Serum samples obtained every day during this immune elimination were also studied for their ability to bind [^{131}I]C1q (●——●). Between day 7 and day 11 there is a significant precipitation of [^{131}I]C1q.

the radiolabeled C1q binding test, a concentration of 100 μg/ml of agg HGG still led to an increased and significant [^{125}I]C1q precipitation of 25%.

By the hemolytic technique, the minimal concentration needed for the detection of anticomplementary activity by agg HGG was found to be similar to that needed for significant [^{125}I]C1q precipitation.

In a control experiment, insoluble immune complexes were prepared by incubating BSA with F(ab')$_2$ fragments from rabbit anti-BSA antibodies. In presence of [^{125}I]C1q the formation of such complexes did not lead to a precipitation of [^{125}I]C1q, even after addition of 2.5% PEG (Table I).

In the presence of EDTA, DNA is known to precipitate C1q and is used for the preparation of purified C1q (15). Furthermore, it is known that DNA, especially SDNA (42), may circulate freely in the blood of patients with SLE. Therefore, it was important to test whether DNA would bind [^{125}I]C1q under the conditions of this test. Increasing concentrations of NDNA between 0.5 and 150 μg/ml were added to NHS and [^{125}I]C1q in the presence of 2.5% PEG, but no significant precipitation of [^{125}I]C1q occurred, with or without addition of 2.5% PEG to the test system at the pH and ionic strength used for this test. A specific [^{125}I]C1q precipitation of only 5% could be observed when SDNA (25 μg/ml) was added to heat-inactivated NHS. This precipitation was eliminated by pretreatment of the serum containing SDNA by pancreatic DNase 1 (0.2 mg/ml, 2 h at 37°C).

The reproducibility of the radiolabeled C1q binding test was evaluated by determining one serum sample of a particular SLE patient 20 times. The intrinsic error of the method was found to be low since the standard deviation of these measurements was less than 3%.

Radiolabeled C1q binding test in experimental immune complex disease. Acute serum sickness was induced by intravenous injection of BSA (200 mg/kg) and trace amounts of [^{125}I]BSA in four rabbits immunized 3 days before with 20 mg of BSA in CFA. As shown in Fig. 4, the elimination of the antigen was quite classical with a disappearance of 99.9% of circulating antigen 14 days after the immunization. Serum samples obtained every day during this immune elimination were studied for ability to bind [^{131}I]-C1q. It was found (Fig. 4) that between day 7 and day 11 there was a significant precipitation of [^{131}I]-C1q. These data are in fact well correlated with the demonstration of globulin-bound BSA in the serum by the Farr technique (32). Therefore, the precipitation of radiolabeled C1q in presence of PEG seems adequate for the detection of in vivo formed immune complexes.

Radiolabeled C1q test in patients with SLE. 52 sera from 22 patients with SLE were assayed for their binding of [^{125}I]C1q. It was found that the SLE patients had a significantly increased [^{125}I]-C1q binding when compared to the values obtained with 48 NHS ($P < 0.001$). The mean value (±1 SD) for the SLE group was 42±27% [^{125}I]C1q ppt. The mean±1 SD of the 48 NHS was 16±6% [^{125}I]C1q ppt.

The single [^{125}I]C1q precipitation values were also grouped according to the hemolytic activity of the same serum. Sera with lytic activities (CH_{50}) below the 2 SD range of a normal control group were attributed

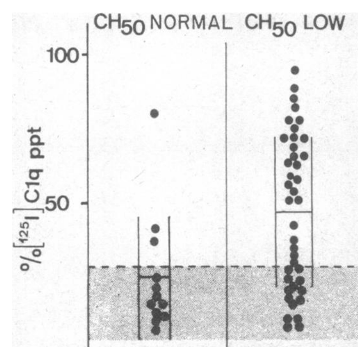

FIGURE 5 [^{125}I]C1q binding by 52 sera from patients with SLE. Shadowed area indicates the mean±2 SD range of the values obtained with 48 NHS. A significant increase in [^{125}I]C1q binding is observed for the SLE patients ($P < 0.001$). The difference between the "CH_{50} low" and the "CH_{50} normal" groups is also significant ($P < 0.005$).

TABLE II

Comparison of [^{125}I]C1q Binding with Various Parameters of SLE by Regression Analysis with the Method of Least Squares

[^{125}I]C1q binding compared to:	Regression coefficient	Confidence Limit for r	t test	Probability of t
Hemolytic complement activity	0.437	±0.187	3.919	<0.001
NDNA binding	0.126	±0.175	1.212	>0.05
SDNA binding	0.216	±0.153	2.372	<0.05
Serum IgG (mg/100 ml)	2.283	±5.555	0.692	>0.40

to the "CH_{50} low" group, and sera with lytic activities within this range were attributed to the "CH_{50} normal" group. The results are shown in Fig. 5. Significantly increased [^{125}I]C1q precipitation is observed within the group with low CH_{50} values when compared to both the CH_{50} normal group ($P < 0.005$) and the group of the normal blood donors ($P < 0.0005$).

Similar results can be observed when comparing [^{125}I]C1q binding values with other parameters of SLE such as antibody activity to NDNA and SDNA (Table II). This table presents the results of a regression analysis done with the entity of studied sera. It confirms the data obtained in Fig. 5. Furthermore, it appears that there is a positive correlation between [^{125}I]C1q precipitation and activity against SDNA. The serum IgG level, however, is completely unrelated to the [^{125}I]C1q precipitating activity.

These results were corroborated in serial studies of four particular patients suffering from SLE. Generally, it was found that the [^{125}I]C1q precipitation was clearly related to the disease activity. One typical example is the history of a 15-yr-old girl suffering from SLE and lupus nephritis. During the evolution (Fig. 6) there was first a phase of acute disease followed by improvement under therapy with steroids and 6-mercaptopurine. During this phase, one could observe a decrease of anti-NDNA antibodies and a normalization of the hemolytic complement activity. Simultaneously, the [^{125}I]-C1q binding was significantly elevated at the beginning and then decreased to normal values. Later, there was a relapse of the disease with a change for the worse in all serological parameters and an increase in [^{125}I]-C1q precipitation. During the recent improvement there was again a decrease in [^{125}I]C1q precipitation which paralleled the decrease of anti-NDNA antibodies and proteinuria. The hemolytic complement activity was raised to normal values during this phase. Serum IgG levels began to decrease at the end of 1972 and stayed low from November 1972 onwards. However, a very high [^{125}I]C1q precipitation persisted until February 1973. Serum IgM and IgA levels were controlled during the whole observation period and were found to be in the normal range.

To further define the serum factors responsible for [^{125}I]C1q precipitation in the serum of SLE patients, sera binding > 80% of [^{125}I]C1q were fractionated on Sephadex G-200 columns. The fractions obtained were then assayed in the [^{125}I]C1q binding test by maintaining the final protein concentrations constant by using NRS as a diluent. Fig. 7 shows a representative experiment. It can be seen that the fractions corresponding to the large molecular elution peak are found to precipitate increased percentages of [^{125}I]C1q. Testing of the macromolecular exclusion peaks of three NHS in an identical procedure resulted in [^{125}I]C1q precipitation percentages which were increased by only 4–6% as compared to the control values obtained with NRS.

Since it is known that reduction and alkylation of complement-fixing 7S antibody or agg HGG leads to destruction of their complement fixing ability, we have

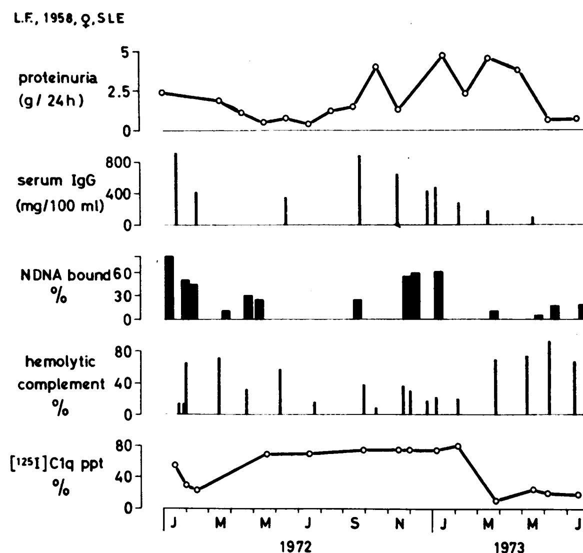

FIGURE 6 Comparison of the evolution of various parameters for SLE disease activity in the follow-up study of a 15-yr-old girl. Periods with normal hemolytic complement activities are characterized by low [^{125}I]C1q binding activities. Low CH_{50} activity, proteinuria, and high titered anti-DNA antibodies are further characterizing periods associated with increased [^{125}I]C1q binding.

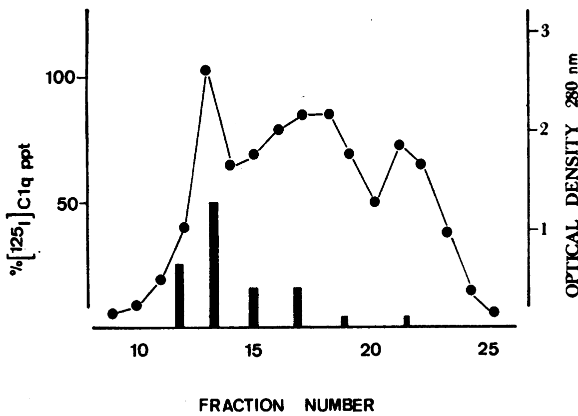

FIGURE 7 Fractionation pattern with OD measurement at 280 nm (●——●), obtained with one serum from a patient with high [125I]C1q binding on Sephadex G-200 (bars, right ordinate). The fractions were pooled according to the molecular size and were assessed for [125I]C1q precipitation. Strongly increased [125I]C1q binding is seen in the macromolecular elution zone.

tested the [125I]C1q precipitating activity of eight SLE sera before and after treatment with 0.2 M mercaptoethanol. The mean value of the [125I]C1q ppt was decreased from 38% to 24% after treatment with mercaptoethanol. The same procedure led to a decrease from 82 to 55% [125I]C1q ppt in presence of 0.5 mg/ml agg HGG solution.

Radiolabeled C1q test in carriers of the HB-Ag. 54 sera from healthy carriers or patients with the HB-Ag were investigated for their [125I]C1q precipitating activity.

A substantial difference was found in the [125I]C1q precipitation by sera from patients suffering from hepatitis as compared to healthy carriers of the HB-Ag ($P < 0.025$) and a significant difference when compared to normal blood donors ($P < 0.0005$). The group of healthy carriers did not exhibit increased [125I]C1q precipitation when compared to normal human donors ($P > 0.4$). Seven out of 24 cases with acute transient hepatitis and four out of seven cases with chronic

TABLE III

[125I]C1q Precipitation in 24 Patients with Acute Hepatitis and HB-Ag

Patient	Days after onset of jaundice	[125I]C1q ppt	Serum total bilirubin	SGOT*/SGPT	IgG	Histological diagnosis
		%	*mg/100 ml*	*U/liter*	*mg/ml*	
B. J.	3	16‡	6	288/920	ND	AH*
G. N.	3	79	11	472/712	18.2	ND
D. E.	4	28	14	408/880	8.9	AH
M. D.	4	16	5	19/45	10.6	ND
S. A.	4	13	13	1,180/800	12	AH
S. M.	5	10	7	136/219	ND	AH
E. R.	6	49	13	850/840	13.7	AYA
G. A.	6	11	0.8	120/47	ND	ND
C. G.	7	43	10	1,200/1,100	10.0	ND
F. P.	7	17	5	160/340	23	ND
S. N.	7	20	5	356/436	8.6	AH
N. H.	8	23	4	260/608	17.3	AH
B. P.	9	20	13	469/640	ND	AH
G. H.	9	25	15	816/1,080	10.8	AH
C. B.	13	20	15	808/1,100	9.3	AH
I. M.	13	40	9	620/510	16.6	AH
A. R.	14	21	24	620/848	7.5	AH
M. M.	16	18	11	784/1,182	21	ND
G. J.	18	29	16	520/646	6.5	ND
R. A.	19	40	17	164/376	6.8	AH
U. J.	22	9	10	1,200/1,150	7.5	AH
E. R.	25	12	15	425/485	15.0	AYA
E. S.	25	18	11	384/456	11.5	AH
S. J.	30	18	12	390/610	22	ND

* AH, acute viral hepatitis; AYA, acute yellow atrophy; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

‡ Mean±1 SD of 48 NHS = 16±6.

Table IV

[^{125}I]C1q Precipitation in 7 Patients with Chronic Hepatitis and HB-Aq

Patient	[^{125}I]C1q ppt	Serum total bilirubin	SGOT/SGPT*	IgG	Histological diagnosis
	%	*mg/100 ml*	*U/liter*	*mg/ml*	
B. M.	90‡	11	235/95	ND	CH*
B. H.	22	1.3	23/28	ND	CH
G. H.	14	1	17/23	ND	CH
M. E.	46	12	780/800	16.4	CH
P. G.	40	0.5	30/16	23	CH
S. G.	36	13	133/129	12.6	CH
T. M.	27	2	25/32	16	CH

* CH, chronic hepatitis; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.
‡ Mean±1 SD of 48 NHS = 16±6.

persistent hepatitis had increased [^{125}I]C1q precipitation values deviating from the normal mean by more than 2 SD. Each patient's condition is represented in Tables III and IV. As seen in Table III, most of the sera were collected during the icteric phase of the disease. There is no correlation between the increased [^{125}I]C1q ppt in the seven positive sera with the clinical status of the patient. Increased [^{125}I]C1q ppt was found in a high proportion of HB-Ag carriers with chronic hepatitis (Table IV). Patient B. M. died 3 mo after test performance.

In one patient, the C1q-precipitating material was searched for in a sequential study. There was an increase of [^{125}I]C1q ppt at the end of the icteric phase which was simultaneously characterized by the elimination of the circulating antigen (Fig. 8).

There was no significant correlation between the [^{125}I]C1q precipitation and the IgG levels in the corresponding sera as calculated by the method of the least squares (probability of $t > 0.01$).

DISCUSSION

The detection of circulating immune complexes has been performed by a great variety of methods. In part, these methods take advantage of the fact that the immune complexes are macromolecular substances which can be separated from other serum proteins. Both chromatographic separation (10) and ultracentrifugation (11) are based on that principle. Immuno-electron-microscopy has been used for morphological studies (8). A more widely used method for the demonstration of immune complexes is based on their anticomplementary activity (43). Finally, we ourselves have used PEG in order to precipitate immune complexes (22).

A more specific method, however, for the demonstration of immune complexes was developed some years ago by using the property of C1q to interact specifically with complexed antibody (14, 15). In a gel diffusion system, agg HGG give precipitin lines with C1q. Beyond specificity, such a system has the advantage of being relatively independent of the antigen size.

The presently described radiolabeled C1q test is based on the binding of [^{125}I]C1q by immune complexes with subsequent precipitation of complex-bound [^{125}I]-C1q by PEG. The exclusion of complex-bound [^{125}I]C1q from the solvent in presence of this synthetic polymer appears to be due to the large size of the reaction product of [^{125}I]C1q and immune complexes or agg HGG, since these constituents alone are soluble at the PEG concentrations used. The general mechanism of action of PEG is not known but similarities with the modification of protein solubilities in presence of polysaccharides would suggest that a steric exclusion of the macromolecular complex from the domain of the polymer in involved (44).

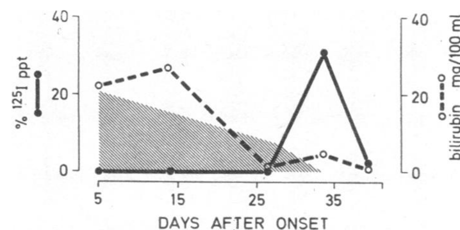

Figure 8 [^{125}I]C1q ppt (●——●) in a patient suffering from hepatitis and carrying the HB-Ag (corrected for nonspecific precipitation). The elimination of the antigen from the circulating blood (shadowed area), as measured by the [^{125}I]Fab anti-HB-Ag antibody precipitation is accompanied by a decrease in total serum bilirubin (○- - -○). Note appearance of C1q-precipitating material during early remission phase.

Precipitation of $[^{125}I]C1q$ by immune complexes was achieved in two different antigen-antibody systems. With either human IgG or BSA as an antigen, insoluble complexes could bind $[^{125}I]C1q$ up to more than 90%. This is proof of preservation of binding capacity of C1q after radioiodination with lactoperoxidase, and correlates well with the results reported by Heusser et al. (23). In large excess of antigen, where the majority of the complexes are soluble, IgG-anti-IgG complexes still strongly fix $[^{125}I]C1q$. One may consider that most of the complexes formed in 50 times IgG excess contain one molecule of anti-IgG antibody and two molecules of IgG (Ag_2Ab). It is likely that most of the antibody reactive sites are bound to antigen in such conditions, since the anti-IgG antibody used in this experiment has a high avidity. The amount of antibody needed for a significant $[^{125}I]C1q$ precipitation in the BSA-anti-BSA system was 10–20-fold greater than the amounts needed in the HGG-anti-HGG system. One might explain this phenomenon either by the fact that in the latter system the antigen itself is an immunoglobulin susceptible to binding C1q after immune aggregation or by the smaller size of the complexes formed in antigen excess in the BSA-anti-BSA system. Soluble immune complexes prepared by separation of any insoluble material by centrifugation had a strong $[^{125}I]$-C1q precipitating property. In the latter experiments, there is only a small percentage of soluble IgG-anti-IgG complexes precipitated by the addition of PEG and these results suggest that only the complexes binding $[^{125}I]C1q$ become less soluble in PEG.

The specificity of the radiolabeled C1q binding test for the Fc part of antibody in immune complexes was evidenced by the inability of antigen-$F(ab')_2$ antibody complexes to lead to a precipitation of $[^{125}I]C1q$ in PEG. Furthermore, the aggregation of immunoglobulins was shown to be a prerequisite for the precipitation of $[^{125}I]C1q$ in this test. While unspecific fixation of C1q to DNA is known to occur under certain conditions, such binding could not be observed under the conditions of the test. On the other hand, SDNA was found to precipitate a small percentage of $[^{125}I]C1q$. This fact might be of particular importance since it is known that small amounts of free DNA are present in the sera of some subjects (42). However, on the basis of the results reported here, one cannot exclude a hypothetical reactivity of $[^{125}I]C1q$ with circulating antibodies to altered C1q, although such antibodies have not been demonstrated as yet. Besides, one should be aware that several anionic substances other than DNA can bind C1q nonspecifically and therefore in high concentration they may act as immune complexes.

Prior reduction and alkylation of SLE sera or of agg HGG did reduce significantly the $[^{125}I]C1q$ ppt. Therefore, such treatment may be used to further confirm the immune complex nature of the C1q binding material in patients' sera.

It appeared that the use of trace amounts of radiolabeled C1q instead of high concentrations of cold C1q resulted in a better sensitivity of the $[^{125}I]C1q$ precipitation test than that of the C1q gel diffusion test reported earlier (15). Furthermore, gel diffusion systems only allow for semiquantitative results; whereas, radioimmunoassays give more quantitative data. This might be useful in follow-up studies of patients and appraisals of the efficacy of immunosuppressive treatment. The radiolabeled C1q test is comparable to that of the hemolytic evaluation of anticomplementary activity in terms of sensitivity. However, various inhibitors acting at different stages of the complement system may be present in patients' sera. Such inhibitors are not easily distinguished from circulating immune complexes when one is using the hemolytic evaluation of the anticomplementary activity. In contrast, most of the inhibitors do not positively influence the $[^{125}I]C1q$ precipitation.

The reproducibility of the test is good, although it is known that C1q molecules are subject to spontaneous aggregation when in free solution. It is therefore important to centrifuge the $[^{125}I]C1q$ before use for the test and to maintain a convenient protein concentration in the reaction tubes in order to stabilize free $[^{125}I]C1q$.

The results observed with in vitro formed immune complexes inspired us to apply the radiolabeled C1q test to the study of an experimental condition in which soluble immune complexes occur in vivo. It is well known that soluble immune complexes are present in the circulating blood during the phase of immune elimination of the antigen during acute serum sickness (45). Indeed, the $[^{131}I]C1q$ binding was increased in serum samples obtained during this phase of experimental serum sickness. The fact that the $[^{131}I]C1q$ binding in serum reaches a maximum before the complete elimination of circulating antigen suggests that, in this model, the percentage of $[^{131}I]C1q$ ppt is mainly related to the absolute amount of circulating complexes rather than to the relative size of these complexes.

Therefore, the $[^{125}I]C1q$ precipitation assay seemed applicable to the study of human sera in which soluble immune complexes are suspected. The increased $[^{125}I]$-C1q precipitation observed in sera from patients with SLE agrees well with the results obtained with the gel diffusion system described earlier (16). It is likely that DNA-anti-DNA complexes are involved in the pathogenesis of SLE and its renal complications (2). Since DNA has been shown in the circulation of some SLE patients (42), it is conceivable that DNA-anti-DNA complexes might be formed in vivo in the plasma of

SLE patients. However, the exact nature of the material in SLE which binds to C1q remains unknown.

The precipitation of [^{125}I]C1q by sera from SLE patients could be correlated to some parameters of SLE such as hemolytic activity, anti-DNA antibodies, and proteinuria. By far the best correlation exists between the hemolytic complement activity and [^{125}I]C1q precipitation and this might result from a direct action of the C1q-precipitating material on the complement system in vivo. Since the tested sera are heat inactivated, one can largely exclude a competition of intrinsic C1q with the [^{125}I]C1q added for the test and therefore it is unlikely that low levels of C1q in SLE sera would favor the increase in [^{125}I]C1q binding.

It was shown that the increase in [^{125}I]C1q binding in these patients is not related to the level of IgG in serum and it is improbable that a precipitation of [^{125}I]-C1q by IgG aggregates, formed in vitro in presence of an increased concentration of IgG, would occur. It is evident that several patients have a low CH_{50} activity in serum with a [^{125}I]C1q precipitation within the normal range. However, one cannot exclude the possibility of intratissue complement binding in the absence of large amounts of circulating complexes. Moreover, it has been suggested that the synthesis rate of some complement components may be decreased in patients with SLE (46).

Whether the [^{125}I]C1q-precipitating material in SLE plays an important role in the renal disease is unknown. In four cases which were studied in follow-up investigations, the [^{125}I]C1q binding might reflect the severity of the disease as defined by decreased hemolytic complement activity, increased anti-DNA binding activity, and proteinuria.

Previous workers (47) have stressed the point that serum from patients with rheumatoid arthritis contain complexes which are detectable by precipitation with monoclonal rheumatoid factor, but not by precipitation with C1q in gel diffusion. The increased sensitivity obtained by using [^{125}I]C1q and PEG seems to attenuate this paradox. Indeed, [^{125}I]C1q-binding material was detected in approximately two-thirds of patients with seropositive rheumatoid arthritis (48). Besides, the low reactivity of monoclonal rheumatoid factor with SLE sera may reflect the different nature of complexes appearing in SLE and rheumatoid arthritis.

Another application of the in vitro interaction between [^{125}I]C1q and immune complexes has been achieved by studying sera from patients with hepatitis and exhibiting the HB-Ag in their sera. The C1q agar gel precipitation test has been used earlier for the detection of HB-Ag-antibody complexes in patients suffering from polyarteritis nodosa (19). It has been suggested that the HB-Ag virus forms complexes with its specific antibody, either at the site of the injury (49) or in blood (8, 43). This latter supposition is now further strengthened by the results obtained with the presently described radiolabeled C1q binding test on sera of HB-Ag hepatitis patients. Moreover, HB-Ag carriers who are clinically healthy have a [^{125}I]C1q precipitation which is within the normal range with the exception of one case. Although the number of observations in the chronic persistent hepatitis group is much too low to allow for a significant conclusion, the result nevertheless suggests an occurrence of C1q-precipitating material in more chronic cases. This would be consistent with the observations of other investigators who found increased anticomplementary activities more frequently in sera from patients with cirrhosis of the liver than in other individuals (50).

There are four main limitations of the presently described method: (*a*) only immune complexes involving C1q-binding antibodies can be detected; (*b*) hypothetical autoantibodies to C1q could account for false positive results; (*c*) only fresh or freshly frozen serum should be used to avoid the spontaneous formation of immunoglobulin aggregates; (*d*) the test gives no direct indication for the identification of the antigen in the complexes.

At the present time one can consider that the radiolabeled C1q binding test constitutes a sensitive, quantitative, and reproducible tool for the detection of immune complexes. Further efforts will be devoted to the evaluation of the clinical implications of this procedure.

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