Solid-Phase C1q-Binding Fluorescence Immunoassay for Detection of Circulating Immune Complexes

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A fluorescence immunoassay for detection of immune complexes bound to solid-phase C1q was developed. The method was standardized by using human aggregated immunoglobulin G (IgG) to simulate immune complexes. A linear relationship existed between the concentrations of the aggregated IgG standards and the resulting fluorescent intensity. The method was found to be reproducible and capable of detecting as little as 10 μ g of aggregated IgG per ml of heat-inactivated human serum. Antigen-antibody complexes prepared in vitro were detectable from equivalence to moderate antigen excess. Endogenous serum C1q inhibited the binding of aggregated IgG to solid-phase C1q. Pretreatment of test sera with EDTA was ineffective in eliminating this competitive effect. Heating the sera at 56°C alleviated, but did not abolish, interference of endogenous C1q. Elevated levels of immune complexes were detectable in sera from seven of nine patients with systemic lupus erythematosus, provided the samples were heat inactivated before testing. Heparin and DNA were also found to interfere with the detection of aggregated IgG added to human serum. Assay values were falsely decreased due to competitive inhibition by these anions. Lipopolysaccharides from a variety of bacterial preparations produced no detectable interference. A comparative study was conducted on samples that had previously been tested by fluid-phase C1q-binding radioimmunoassay. The two methods were concordant in assigning normal or elevated levels of immune complexes in 70% of the samples tested. This solid-phase fluorescence immunoassay is proposed as a possible alternative to radioimmunoassay for the detection of circulating immune complexes.

Numerous non-antigen-specific methods for the detection of immune complexes have been described (12, 15). Many of these assays utilize the ability of antigen-antibody complexes to combine with the complement component C1q and have achieved widespread use. The majority of C1q-binding assays use radio-labeled components to quantitate immune complexes (22, 26). Such radioimmunological methods are extremely sensitive for detection of biologically important substances, but hazards exist in the handling and disposal of radioactive materials. Recent advances in fluorimmunoassay (FIA) indicate that fluorescent labels are suitable substitutes for radiolabels in many immunoassays (17, 20).

We have developed a fluorescence assay for the detection of immune complexes bound to solid-phase C1q. The method has been adapted from the FIAX system described originally by International Diagnostic Technology (IDT) of Santa Clara, Calif. C1q is adsorbed to the surface of the IDT StiQ sampler, which is then incubated with the diluted test sample. Antigen-

antibody complexes present in the serum bind to C1q via the Fc portion of the immunoglobulin in the complex. After washing, the bound components are then incubated with fluorescein-labeled anti-immunoglobulin G (IgG). Nonreactive substances are washed away, and the remaining fluorescence bound to the solid phase is measured in the FIAX fluorometer. We propose that FIA is an alternative to radioimmunoassay (RIA) for the detection of immune complexes and thus eliminates the use of hazardous radioisotopes.

MATERIALS AND METHODS

Aggregated IgG (AGG). Human IgG (Gammagee; Merck, Sharp & Dohme, West Point, Pa.) was diluted to a concentration of 10 mg/ml in phosphate-buffered saline (PBS), pH 7.2. The preparation was aggregated by heating for 20 min at 63°C and then centrifuged at $1,500 \times g$ for 20 min to remove insoluble aggregates.

Preparation of solid-phase C1q (SP-C1q). StiQ samplers (IDT) were used as the solid-phase matrix. The samplers were placed horizontally in a moist chamber with the polymeric surface upward. Purified C1q, purchased from David Bing at the Center for Blood

Research, Boston, Mass., was diluted to a concentration of 50 $\mu\text{g/ml}$ in PBS. Thirty microliters of the diluted C1q was placed on the cellulose disk of each sampler, using a hand-held pipetter. The samplers were refrigerated overnight in the moist chamber.

Fluorescent-antibody reagent. Purified F(ab')₂ goat anti-human immunoglobulin conjugated with fluorescein isothiocyanate (FITC) was purchased from Kallestad Laboratories, Chaska, Minn. The antiserum was diluted to a concentration of 8 $\mu\text{g/ml}$ in PBS containing 0.125% bovine serum albumin (BSA).

Reaction buffer and wash buffer. PBS, 0.05 M, containing 0.1% Tween 20 and 0.5% BSA, pH 6.4 (PBS-BSA-Tw), was used as sample diluent and initial wash solution for solid-phase components.

Immune complex assay. Assays were carried out in glass tubes (12 by 75 mm), and all incubations were allowed to proceed on an automatic horizontal shaker. IDT samplers coated with 1.5 μg of C1q were individually washed for 30 min in tubes containing 0.5 ml of PBS-BSA-Tw buffer. Blank control samplers for each specimen were also washed in this buffer to coat the exposed polymeric surface with BSA and were carried through the procedure concurrently with the samplers coated with immobilized C1q. The samplers were then transferred to tubes containing 50 μl of AGG standards, controls, or unknown samples and 500 μl of PBS-BSA-Tw buffer and incubated for 2 h at room temperature. All samplers were washed for 30 min in PBS-BSA-Tw and then immersed for 45 min in 0.5 ml of FITC-F(ab')₂ goat anti-human IgG. Unreacted materials were washed away by transferring the samplers to tubes containing 0.5 ml of PBS with 2.0% BSA. After this final 30-min wash, each sampler was then inserted into the sample stage of the FIAX fluorometer, and the fluorescent intensity was measured.

Quantitation of fluorescence. The FIAX fluorometer measures the fluorescence of components bound to the sampler at a wavelength of 540 nm when excited by light at a wavelength of 475 nm (11). The fluorometer was adjusted to zero without a sampler in the sample stage. The 200- $\mu\text{g/ml}$ AGG standard was then inserted, and the fluorometer was manually adjusted to read 190 ± 5 fluorescent signal units (FSU). The remaining standards and unknown samplers as well as the blanks for each specimen were then inserted into the fluorometer, and the FSU of each was recorded. The FSU of the blank sampler for each specimen was subtracted from the FSU of the sampler coated with C1q to obtain a ΔFSU . On linear graph paper, micrograms of aggregated IgG per milliliter was plotted on the x axis, and the ΔFSU value of the AGG standards were plotted on the y axis. The degree of fluorescence was directly proportional to the concentration of aggregates present. Control and patient samplers were compared with the standard calibration curve, and the results were expressed as microgram equivalents of AGG per milliliter.

Sera. Normal human sera (NHS) were collected from apparently healthy hospital personnel after obtaining informed consent. Control sera were prepared by adding AGG to heat-inactivated (30 min at 56°C) NHS. Patients' sera that had been tested in a fluid-phase [¹²⁵I]C1q-binding assay (16) were kindly supplied by Scripps-Miles Immunology Reference Laboratory, La Jolla, Calif. All specimens were divided into

equal portions, stored at -70°C , and thawed just before testing.

Preparation of IgG-anti-IgG complexes. Chromatographically purified human IgG was supplied by Tago Laboratories, Burlingame, Calif. The preparation was centrifuged in a Beckman Airfuge (Beckman Instruments, Inc., Palo Alto, Calif.) for 20 min at $165,000 \times g$ to remove soluble aggregates. Goat anti-human IgG was purchased from Meloy Laboratories (Springfield, Va.), and the specific antibody content was provided by the company. IgG-anti-IgG complexes were prepared by incubating a constant amount of goat anti-human IgG with increasing amounts of monomeric human IgG for 1 h at 37°C followed by a 48-h incubation at 4°C. The precipitin curve was determined (9), and the antigen/antibody ratio at equivalence was found to be 1:2.5. Immune complexes were prepared at four concentrations of goat anti-human IgG: 20, 50, 100, and 500 $\mu\text{g/ml}$. Increasing amounts of human IgG were added to constant amounts of antibody to produce complexes ranging from 8 times antibody excess to 15 times antigen excess as compared with the equivalence ratio. Both the antigen and the antibody were diluted in PBS and incubated together for 1 h at 37°C followed by a 48-h incubation at 4°C. After centrifugation for 20 min at $1,000 \times g$, the supernatants were tested in the immune complex assay.

Preparation of tetanus toxoid-antitetanus toxoid complexes. Purified tetanus toxoid was purchased from Lederle Laboratories, Pearl River, N.Y. The undiluted preparation was dialyzed against PBS for 24 h at 4°C. Protein content as determined by the method of Lowry et al. (13) was 165 $\mu\text{g/ml}$. The immunoglobulin fraction of a human antitetanus antiserum (Hypertet) was purchased from Cutter Laboratories, Berkeley, Calif. This antiserum was diluted in PBS and centrifuged for 60 min at $130,000 \times g$ in a Beckman L5-65 ultracentrifuge to remove aggregated immunoglobulins. Soluble immune complexes were prepared by using a 1:10 and 1:20 dilution of the tetanus antisera. Increasing amounts of toxoid (10 to 165 $\mu\text{g/ml}$) were added to tubes containing constant amounts of the antibody-containing fraction. Both the antigen and the antibody were diluted in PBS and incubated together for 1 h at 37°C followed by incubation for 4 days at 4°C. The tubes were then centrifuged for 30 min at $1,000 \times g$, and the supernatants were tested for the presence of antigen or antibody by Ouchterlony analysis and visually by the Swift-Wilson-Lancefield capillary tube test (14). Equivalence was determined as that tube in which neither antigen nor antibody could be detected. The supernatants were then tested in the immune complex assay.

Potential interfering substances. Double-stranded (ds) DNA (calf thymus DNA, type I) was purchased from Sigma Chemical Co., St. Louis, Mo. Single-stranded (ss) DNA was prepared by heating the native DNA at 100°C for 10 min at a concentration of 0.5 mg/ml in PBS, followed by immediate cooling in an ice bath. Liqueamin sodium heparin was purchased from Organon, Inc., West Orange, N.J.

Lipopolysaccharides (LPS) from the following bacterial preparations were purchased from Sigma: phenol extract of *Salmonella minnesota*, *S. typhosa*, *S. typhimurium*, and *Escherichia coli* O111:B4; trichloroacetic acid extract of *S. enteritidis*, *S. typhosa*, and *S. typhimurium*. The trichloroacetic acid extracts of *S.*

FIG. 1. Linearity and precision of the standard curve. Each point represents the mean of 30 separate determinations of each AGG standard. Vertical bars represent standard deviation from the mean.

minnesota 9700 and of *E. coli* O111:B4 were purchased from Difco Laboratories, Detroit, Mich. The lyophilized preparations were diluted in normal saline and heated to boiling for 10 min to ensure solubility.

RESULTS

Linearity and precision of the standard curve. Standard curves were constructed by assaying AGG at concentrations of 10, 25, 50, 100, and 200 μg/ml in PBS. To determine linearity and reproducibility the ΔFSU values of 30 separate curves were examined. The results shown in Fig. 1 illustrate the mean and standard deviation of the fluorescence obtained with each AGG standard. Least-squares linear regression analy-

sis resulted in a correlation coefficient of 0.996, and the slope of the curve was 0.93. These results indicated that a nearly perfect relationship existed between the concentration of AGG and the degree of fluorescence produced, and that the binding of AGG to SP-C1q followed a linear dose-response curve.

Assay of complexes formed in vitro. IgG-anti-IgG complexes were prepared by using human monomeric IgG and goat anti-human IgG antibody. We recognized that quantitation of these prepared complexes would be based on the binding of FITC-anti-human IgG to the antigen (human IgG) bound in the complex. Therefore, in addition to verifying the detection of antigen-antibody complexes, we assessed the adaptability of this assay as an antigen-specific method. Controls which contained antibody or antigen alone were included. The goat anti-human IgG antibody alone produced fluorescence equivalent to that of the buffer blank. The fluorescence of the human monomeric IgG alone was minimal at all concentrations tested (Fig. 2). Soluble complexes were detected from equivalence to five times antigen excess at all antibody concentrations. Complexes in antibody excess were not detected.

Soluble tetanus toxoid-antitoxoid complexes were tested in a similar manner, and controls were included which contained antigen or antibody alone. The tetanus toxoid antigen alone produced fluorescence no higher than that of the buffer blank, and fluorescence of the antitetanus antisera was minimal (Fig. 3). Soluble complexes were detected from equivalence to three times antigen excess. Again, complexes in antibody excess were not detected.

FIG. 2. Detection of soluble IgG-anti-IgG complexes prepared in vitro. Complexes were formed with 20 (Δ), 50 (▲), 100 (○), and 500 (●) μg of goat anti-human IgG antibody/ml and varying concentrations of human monomeric IgG. The antigen-antibody ratio at equivalence was approximately 0.4. The control values shown are the resulting FSU of human monomeric IgG alone at the highest concentration used in each assay: 120 (Δ), 300 (▲), 600 (○), and 3,000 (●) μg/ml.

FIG. 3. Detection of soluble tetanus toxoid-anti-toxoid complexes prepared *in vitro*. The complexes were formed with a 1:10 (●) and 1:20 (Δ) dilution of human antitetanus antiserum and varying concentrations of purified tetanus toxoid. The concentration of antigen compared with equivalence is expressed as equivalent amount. For the 1:10 and 1:20 antibody dilutions, this was 40 and 25 μg of toxoid/ml, respectively. The control values shown are the resulting FSU of the 1:10 (●) and 1:20 (Δ) antibody dilutions alone.

Effects of EDTA and 56°C heat inactivation on C1q binding of AGG in human serum. Preliminary investigations demonstrated that addition of exogenous C1q to sera containing AGG inhibited binding of AGG to SP-C1q. It was apparent that test samples must be treated to eliminate this competitive effect. The commonly used techniques are addition of EDTA or heating the test sera at 56°C for 30 min. An experiment was designed similar to one described by Zubler et al. (26) to compare the effects of EDTA and heating on the binding of AGG to SP-C1q and to determine the appropriate handling of test samples. The experimental design was as follows.

(i) AGG was added to untreated NHS at a concentration of 200 $\mu\text{g}/\text{ml}$. After a 30-min incubation at 37°C, portions were diluted 1:2 in either PBS or 0.3 M EDTA, pH 7.5. The final concentration of AGG in the NHS was then 100 $\mu\text{g}/\text{ml}$. Each sample was again divided into portions and incubated for 30 min at either 37 or 56°C.

(ii) NHS was diluted 1:2 in 0.3 M EDTA, pH 7.5 (NHS/EDTA). Then AGG was added at a final concentration of 100 $\mu\text{g}/\text{ml}$ followed by incubation for 30 min at 37°C. Portions were further incubated for 30 min at either 37 or 56°C.

(iii) NHS was heat inactivated for 30 min at 56°C (NHS-56°C). AGG was added at a concentration of 200 $\mu\text{g}/\text{ml}$, and the sample was incubated for 30 min at 37°C. Portions were diluted 1:2 in either PBS or 0.3 M EDTA, pH 7.5. The final concentration of AGG present in the NHS-

56°C was thus 100 $\mu\text{g}/\text{ml}$. Each portion was then incubated for 30 min at 37°C.

(iv) Each of the above samples was tested in the immune complex assay. For all of the conditions, controls without AGG were also tested.

The results are shown in Fig. 4. Addition of EDTA to NHS either before or after the addition of AGG did not eliminate the inhibitory effect of endogenous serum C1q. After incubation of AGG with NHS or NHS/EDTA, heating at 56°C partially restored the binding of AGG with SP-C1q. It is apparent that heating did not destroy all complex-bound C1q but did allow for significantly greater binding. Detection of AGG added to NHS-56°C was essentially the same when the sample was diluted in PBS or EDTA, suggesting that EDTA itself was not adversely affecting the reaction. The C1q-binding activity of the negative controls is shown for each sample. Heating NHS at 56°C resulted in only a slight increase in C1q binding in this assay. Therefore, it appeared that the increased detection of AGG after heat inactivation of positive samples was due to the release of bound endogenous C1q rather than nonspecific aggregation of immunoglobulin.

Effect of 56°C heat inactivation on the detection of immune complexes in sera of SLE patients and normal controls. Serum samples from nine systemic lupus erythematosus (SLE) patients and nine normal individuals were tested in the immune complex assay before and after heating for 30 min at 56°C (Fig. 5). All sera from patients with SLE contained significant levels of antibody to ds DNA (>25 ng of DNA bound/25 μl of serum) when tested by RIA. A mean increase of 6.0 μg equivalents (eq) of AGG/ml was observed in the sera of normal individuals after heat treatment. The mean increase after heating sera of SLE patients was 16.4 μg eq of AGG/ml. This increase was more than 2 standard deviations above the mean increase seen in the normal controls, suggesting that the increased C1q-binding activity was not due merely to nonspecific aggregation alone.

Subsequently, 54 human serum samples obtained from apparently healthy donors were heated for 30 min at 56°C and assayed in triplicate. The mean value obtained was 15.98, and 1 standard deviation was 5.85. To determine the upper limit of normal, the data were ranked from low to high, and the 95% confidence value was calculated. The normal range was found to be 1 to 25 μg eq of AGG/ml.

Detection of AGG in the presence of NHS. The accuracy and precision of this method were studied by adding AGG to heat-inactivated NHS. Controls were prepared to contain approximately 10, 50, and 150 μg of AGG/ml and were tested in triplicate in 10 separate runs. Serum without added aggregates was included

FIG. 4. Effects of EDTA and 56°C heat inactivation on C1q binding of AGG in NHS. Symbols: □, Specific C1q-binding activity of each sample; ■, C1q-binding activity of serum controls without AGG.

with each determination, and the fluorescence of this negative sample was subtracted from that of the serum containing AGG. There was good agreement between the measured and expected concentrations of AGG at all levels, and the results were reproducible (Table 1).

Intra-assay precision was assessed by preparing controls to contain 25, 100, and 150 μg of AGG/ml. Table 2 illustrates good reproducibility when 20 replicates of each control were tested.

Effects of potential interfering substances. The susceptibility of this method to interference by C1q-reactive materials was assessed by a series of competitive inhibition experiments. Fixed volumes of potential interfering substances were added in various concentrations to portions of controls containing known amounts of AGG. Percentage of inhibition was calculated by comparing the amount of AGG detected in the presence of each substance with the amount detected when only PBS had been added to the positive control.

(i) **Bacterial LPS.** Five different types of LPS prepared by two different purification procedures were examined. Each preparation was added at a final concentration of 200 $\mu\text{g}/\text{ml}$ to

sera containing AGG. In all instances, 95% or more of the AGG could be detected only in the presence of 200 μg of LPS per ml.

(ii) **ds and ss DNA.** Increasing concentrations of ds DNA (1.0 to 300 $\mu\text{g}/\text{ml}$) or ss DNA (0.5 to 100 $\mu\text{g}/\text{ml}$) were added to portions of serum containing 50 μg of AGG/ml. As little as 5 μg of ss DNA and 30 μg of ds DNA per ml interfered in this assay (Fig. 6).

(iii) **Heparin.** Increasing concentrations of heparin (1.0 to 200 U/ml) were added to sera containing 70 or 150 μg of AGG/ml. Inhibition was detectable at heparin concentrations of 5 U/ml (Fig. 7). Extremely high concentrations of heparin were required to totally abolish the binding of AGG to SP-C1q.

Comparison of the solid-phase FIA with a fluid-phase RIA. Sixty-eight serum samples were first tested for immune complexes at Scripps-Miles Immunology Reference Laboratory by the [^{125}I]C1q-binding assay as described by Nydegger et al. (16). The specimens were heat inactivated, tested, and frozen at -70°C before being shipped to this laboratory. Upon receipt, the specimens remained frozen at -70°C until being tested in the solid-phase FIA. On the basis of

FIG. 5. Effect of 56°C heat inactivation on the detection of immune complexes in sera of SLE patients and normal controls. Serum samples were tested in the native state and after heating for 30 min at 56°C. Normal, borderline, and positive ranges were delineated after establishing immune complex levels present in healthy individuals.

positive and negative results, the two methods showed 70% agreement and no significant difference at the 80% confidence interval (Table 3).

DISCUSSION

Results of this study suggest that FIA is a useful alternative method for quantitation of immune complexes bound to sp-C1q. The FIAX system used in this investigation is costly due to the plastic samplers used as the solid-phase matrix. In addition, the system requires a specialized fluorometer to accommodate the sampler. Nevertheless, the techniques described in this study should be readily adaptable to other

solid-phase surfaces, allowing for quantitation in conventional fluorometers.

As with most C1q-binding assays, AGG preparations were used to standardize the method. The aggregates could be stored at -70°C for up to 4 weeks and consistently gave a highly reproducible standard curve. The curve was linear (slope, 0.93), and the fluorescent intensity correlated well with the concentration of AGG introduced into the system ($r = 0.996$).

The reproducibility and sensitivity of this assay were assessed by adding AGG to heat-inactivated NHS. There was good agreement between the measured and expected concentrations of AGG at all levels, and this assay proved capable of detecting immunoglobulin aggregates at concentrations as low as 10 $\mu\text{g/ml}$. The absolute amount of AGG detectable in the volume of serum selected for the test was 0.5 μg .

The FIA was applied to the detection of in vitro-formed IgG-anti-IgG and tetanus toxoid-antitoxoid soluble complexes. Detection of these complexes was very efficient in the range of equivalence to moderate (three to five times) antigen excess but appeared less sensitive with small complexes formed in extreme antigen excess. Complexes in antibody excess were not detected. Thus, this method may be applied to the detection of clinically significant immune complexes. The antigen-antibody ratio is an important factor in determining the fate and biological activities of immune complexes. Slight to moderate antigen excess results in complexes of intermediate size which are large enough to activate complement but are not readily recognized and cleared from the circulation by phagocytic cells (25). Therefore, these complexes may have the greatest pathogenic potential, and we have demonstrated that they are detectable with the FIA.

The flexibility of this method was demonstrated by assaying preformed IgG-anti-IgG immune complexes. Quantitation of these complexes was based on the binding of FITC-anti-IgG to the antigen (human IgG) bound in the complex. This immunoassay may thus be adapted to an antigen-specific method by using the appropriate

TABLE 1. Accuracy and interassay precision

Expected ($\mu\text{g/ml}$) ^a	Detected ($\mu\text{g/ml}$) ^b			
	Mean	Range	SD	CV (%)
10	13	9-18	2.7	20.9
50	64	59-70	3.7	5.8
150	152	136-171	11.9	7.8

^a The indicated concentrations of aggregated IgG were added to heat-inactivated normal human serum.

^b Values listed are the results of 10 determinations on 10 separate days. CV, Coefficient of variation.

TABLE 2. Intra-assay precision

Expected ($\mu\text{g/ml}$) ^a	Detected ($\mu\text{g/ml}$) ^b			
	Mean	Range	SD	CV (%)
25	25	21-31	2.7	10.8
100	92	87-100	4.3	4.7
150	164	127-202	20.9	12.7

^a The indicated concentrations of aggregated IgG were added to heat-inactivated normal human serum.

^b Values listed are the results of 20 replicates assayed in the same run. CV, Coefficient of variation.

FIG. 6. Effect of ss and ds DNA on the binding of AGG to sp-C1q. Varying concentrations of ss (●) or ds (▲) DNA were added to serum containing 50 μ g of AGG/ml. Percentage of inhibition was calculated by comparison with aggregated IgG detected in the absence of inhibitors.

fluorescein-labeled antibody. Similarly, other classes or subclasses of antibodies bound in immune complexes may be identified by altering the FITC-labeled probe accordingly.

Endogenous serum C1q was found to inhibit the binding of immune complexes and AGG to SP-C1q. This is not surprising, since most C1q-binding assays are similarly affected (19, 21). Pretreatment of test sera with EDTA is a technique used by others (8, 26) to eliminate endogenous C1q interference, but was found to be ineffective in our assay. EDTA chelates calcium necessary for integrity of the C1qrs complex. However, calcium is not needed for binding of C1q with complexed immunoglobulin. Apparently, addition of EDTA in this solid-phase assay will not "unmask" immune complexes which have bound complement *in vivo*.

FIG. 7. Effect of heparin on the binding of AGG to solid-phase C1q. Various concentrations of heparin were added to serum containing 70 (○) or 150 (▲) μ g of AGG/ml. Percentage of inhibition was calculated by comparison with the amount of AGG detected in the absence of heparin.

Heating the sera at 56°C before testing under the described conditions was found to be essential, even though we recognize that there are problems created by this treatment. Heat inactivation of the test sera after addition of AGG partially destroyed the inhibitory effect of endogenous C1q. However, the levels of detection were somewhat lower than when AGG was added to preheated sera. These findings are consistent with those of Teppo and Wager (24), who reported that free C1q is more heat labile than C1q bound to immune complexes. Therefore, the sensitivity of the FIA for detecting low levels of immune complexes which have fixed complement *in vivo* may be somewhat diminished.

Heating sera at 56°C before detecting immune complexes has often been criticized. Some investigators have found false-positive results due to heat-induced aggregation of serum immunoglobulins (5). Other studies have shown that heating test sera may decrease the ability of preexisting complexes to bind exogenous C1q (19, 26). When sera from normal individuals were tested in the FIA, no significant evidence

TABLE 3. Comparison of the solid-phase FIA and a fluid-phase RIA for the detection of immune complexes^a

Assay	No. positive	No. negative	Total
Solid-phase FIA	25	43	68
Fluid-phase RIA	39	29	68
Total	64	72	136

^a Positive indicates abnormal levels of immune complexes for a given assay. Negative indicates normal levels of immune complexes for a given assay. $\chi^2 = 5.0$; $P < 0.025$

for heat-induced aggregation was observed. However, when sera from patients with SLE were tested, this assay was able to detect complexes in seven of nine patients if the sera were heat inactivated before testing. The increased levels of complex detection were more than 2 standard deviations greater than that seen with normal sera and thus could not be explained by nonspecific aggregation. No decrease in C1q binding was observed in any of the samples after heating, which indicates that complexes were not destroyed by this treatment.

The specificity of this assay was investigated by competitive inhibition studies. Since this method uses a fluorescein-labeled anti-IgG probe, non-immunoglobulin substances that bind C1q will not produce false-positive results. However, if test sera contain antigen-antibody complexes as well as certain other C1q-reactive substances, assay values may be falsely decreased due to competitive inhibition.

Although LPS has been shown to activate C1q (4), concentrations as high as 200 $\mu\text{g/ml}$ did not interfere with the detection of AGG.

Single-stranded DNA and, to a lesser extent, ds DNA were found to inhibit binding of AGG with SP-C1q. This was probably the result of a firm ionic interaction between the positively charged C1q molecule and these anions (3). In addition, the strength of binding to C1q may be dependent on molecular size (10). High-molecular-weight substances such as DNA may be capable of interacting with several C1q-binding sites simultaneously or may simply induce steric hindrance. The reaction conditions of this assay included low-ionic-strength buffer to obtain maximal sensitivity for complexes of IgG. Most anionic substances also bind most strongly with C1q under this condition (5, 10). Possibly, the inhibitory capacity of anionic substances may be decreased by performing this assay at higher ionic strength.

Undoubtedly, interference by such anions is a limitation to the specificity of this method, but it is one that is shared by most immune complex assays using C1q. One major difference, however, is that in other C1q-binding assays, with the exception of the solid-phase method described by Hay et al. (8), the interference by DNA may be manifested as falsely elevated levels of immune complexes (12). Therefore, these techniques might not discriminate between free DNA antigen alone and immune complexes containing DNA. Of the existing methodologies using C1q, it is possible that only direct C1q-binding assays using labeled anti-immunoglobulin can accurately distinguish clinically significant immune complexes from DNA. This suggestion may partially explain the finding of Abrass et al. (1) that the solid-phase C1q-binding

RIA correlated better than the fluid-phase C1q assay with clinical activity in SLE. However, the fact that low levels of complexes may go undetected in the presence of DNA cannot be ignored. Detailed clinical studies were not conducted in our laboratory, but sera from seven of nine SLE patients tested were shown to have elevated levels of immune complexes. These results are consistent with those found in extensive studies of SLE (1, 16, 18). In pathological conditions, antigen-antibody ratios may change daily (7). Interpretation of immune complex assay results must necessarily take all these factors into consideration.

Heparin was also found to inhibit the binding of AGG to SP-C1q. As with DNA, the effect of this polyanion is probably related to negatively charged groups interacting with the cationic C1q (2). Competitive inhibition studies demonstrated that approximately 5 U of heparin per ml of serum was required to interfere with the detection of AGG. The primary significance of this finding is that samples to be tested by this method must not be collected in heparinized tubes. Although it has not been verified, sera from patients undergoing carefully controlled heparin therapy may still be suitable for testing by this method. Patients receiving therapeutic heparin doses generally have plasma levels of 0.1 to 0.5 U of heparin/ml (6, 23). However, heparin absorption and excretion rates vary for each individual, and again, potential interference must be considered when this C1q-binding assay is used for detection of immune complexes.

A comparative study was performed on 68 samples that had previously been tested by fluid-phase C1q-binding RIA. The two methods were concordant in assigning normal or elevated levels of immune complexes in 70% of the samples tested. Our data indicate that the fluorescence method is subject to the same types of problems common to most C1q-binding assays but suggest that the technique may be as reliable in quantitation of immune complexes as other assays currently available. Investigations are in progress to find alternatives to heat inactivation and to minimize the effects of interfering substances. However, the primary significance of this report is that FIA presents an alternative to RIA for the detection of immune complexes.

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