C1q DEVIATION TEST FOR THE DETECTION OF IMMUNE COMPLEXES, AGGREGATES OF IgG, AND BACTERIAL PRODUCTS IN HUMAN SERUM*

By A. T. SOBEL,‡ V. A. BOKISCH,§ AND H. J. MÜLLER-EBERHARD‖

(From the Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

Participation of complement in the pathogenesis of a variety of immunologic diseases has been established. Consequently, immune complexes and other complement activating substances have been searched for in serum and other body fluids of respective patients. Agnello, Winchester, and Kunkel have utilized isolated C1q for the detection of C1q-precipitable substances in pathological sera and joint fluids (1, 2). In pursuing the approach of these authors it was possible to develop a procedure which is considerably more sensitive than the C1q precipitin method.

This procedure is based on the inhibition by immune complexes of radiolabeled C1q uptake to sensitized erythrocytes. The degree of inhibition can be accurately quantitated. In the following we describe the development of the C1q deviation test, its sensitivity with respect to different C1q reactants, and its application to the exploration of the C1q-reactive material in pathological sera.

Materials and Methods

C1q. Highly purified C1q was isolated from fresh human serum as previously described (3). The isolated protein was stored at a concentration of 1 mg/ml in 0.1 M phosphate buffer, pH 7, at −70°C until used.

C1q was labeled with ^{125}I according to the method of McConahey and Dixon (4). Specifically, 0.6 mCi of carrier free ^{125}I (ICN Laboratories, Irvine, Calif.) and 10 μg of chloramine T were added to 1 mg of C1q. The mixture was stirred for 10 min at 4°C and the reaction was stopped by addition of 10 μg of sodium metabisulfite. The labeled protein was dialyzed three times against 10 liters of 0.1 M phosphate buffer, pH 7.2, for 36 h at 4°C and then subjected to ultracentrifugation in a Spinco no. 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 35,000 rpm and 4°C for 60 min. Uptake of ^{125}I was approximately 50%, the specific radioactivity ranged between 0.25 and 0.4 μCi/μg protein. Bovine serum albumin (BSA)[1] at a concentration of 1 mg/ml was added before storage at

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Present address: Service de Nephrologie, Hopital Henri-Mondor, 94010-Creteil, France.

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‖ Cecil H. and Ida M. Green investigator in Medical Research, Scripps Clinic and Research Foundation.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; E, nonsensitized sheep erythrocytes; EA, sensitized sheep erythrocytes; GVB, gelatin Veronal buffer; SVB, sucrose Veronal buffer.

$-70°C$. Thawed (^{125}I) C1q was again centrifuged at 13,000 *g* for 5 min in a Beckman microfuge 152 before use.

Sensitized Sheep Erythrocytes. Sheep erythrocytes (E) were purchased from Colorado Serum Co. Laboratories, Denver, Colo., rabbit antiserum to sheep erythrocytes (A) from Becton, Dickinson and Co., Cockeysville, Md. Three types of sensitized sheep erythrocytes (EA) were prepared: cells were sensitized either with unseparated antiserum, the IgG fraction, or the IgM-containing fraction of the antiserum. In each instance the optimal amount of antibody was determined as follows: cells were incubated at 37°C for 30 min with various dilutions of the antibody source in isotonic Veronal buffer, pH 7.5, containing 0.1% gelatin (GVB), washed, and tested for the capacity to bind [^{125}I]C1q as will be described below. For sensitization of E the dilution of antibody source was chosen which allowed uptake of 40–60% of the radiolabeled C1q offered. The reaction volume was 450 μL, the amount of [^{125}I]C1q 1 μg, and the number of EA 4×10^8.

C1q-Reactive Materials. Soluble IgG aggregates were prepared by heating of solutions of Cohn Fraction II (Lederle Laboratories, Pearl River, N.Y.), as previously described (5). The preparations containing 30 mg protein per ml were stored at $-70°C$ and after thawing, centrifuged before use at 2,000 rpm for 15 min in an International refrigerated centrifuge (International Scientific Instruments Inc., Mountain View, Calif.). Only the upper two-thirds of the tube content was utilized.

Soluble immune complexes were prepared as follows. The IgG fraction of rabbit anti-BSA antiserum was purified by precipitation with 50% saturated ammonium sulfate followed by DEAE cellulose chromatography. The IgG fraction was concentrated to 5 mg protein per ml, dialyzed against phosphate-buffered saline, absorbed with EA, and centrifuged at 45,000 rpm for 60 min in a Spinco ultracentrifuge using an SW 50 rotor. To 0.2 ml of rabbit IgG was added 0.2 ml of BSA solution containing 10 μg to 10 mg/ml of protein. After 1 h at 37°C and 16 h at 4°C the precipitates were removed by centrifugation, washed, and dissolved in 1 ml 0.1 M NaOH for protein determination. The supernates were tested for their ability to bind C1q and to consume serum hemolytic activity.

The following microbial substances were used: (*a*) TCA extract of *Escherichia coli* 08 cell walls; (*b*) TCA extract of *E. coli* 0136 cell walls; (*c*) TCA extract of *E. coli* 0115 cell walls; (*d*) staphylococcus G7 slime layer; (*e*) TCA extract of staphylococcus Copenhagen; (*f*) capsular carbohydrate of pneumococcus R3 type 83; (*g*) slime layer of staphylococcal phage type 80; (*h*) TCA extract of streptococcus group L cell walls; (*i*) lipopolysaccharide *Salmonella typhosa* 0901; and, (*j*) Levan from *Bacillus subtilis*. Substances (*a*) through (*h*) were kindly made available by Dr. Walter Karakawa, Department of Microbiology, Pennsylvania State University, (*i*) was purchased from Difco Laboratories, Detroit, Mich. and (*j*) was kindly provided by Dr. John Spizizen, Department of Microbiology, Scripps Clinic and Research Foundation.

Calf thymus DNA was obtained from Calbiochem, San Diego, Calif. and was dissolved in saline over a 48-h period at a concentration of 1 mg/ml. Denatured DNA was prepared from the above solution by boiling for 10 min. DNAse was purchased from Calbiochem and used at a concentration of 0.2 mg/ml. 5 mg of native DNA was treated with 1 mg of DNAse for 3 h at 20°C.

Buffers. Veronal buffer (VB) contained 8.383 g of NaCl, 0.3 g of Na barbital and 0.46 g of barbituric acid per liter, and GVB was prepared by addition of 1 g of gelatin to 9 liters of VB. Sucrose Veronal buffer (SVB) contained 9.25% ultra-pure sucrose (Becton, Dickinson and Co., Cockeysville, Md.), 0.025 M sodium barbital, 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++}. The pH was adjusted to 7.2 with HCl, and the conductance to 3 mmho/cm with isotonic saline. SVB-EDTA contained 0.0125 M EDTA instead of Ca^{++} and Mg^{++}.

Serum Reagents. Normal human serum was obtained from healthy donors and stored at $-70°C$. Sera from patients with Dengue fever were collected as previously described (6) and stored at $-70°C$. Before testing, the sera were centrifuged at 2,000 rpm for 30 min.

Procedure of the C1q Deviation Test. The test consists of the following steps: (*a*) 50 μl of serum, 100 μl of saline, and 100 μl of SVB are mixed in a plastic test tube, the pH being 7.2 and the conductivity 9 mmho/cm; (*b*) the diluted serum sample is heated at 56°C for 30 min; (*c*) 1 μg of [^{125}I]C1q contained in 10 μl solution is added to the sample and the mixture is held at 20°C for 15 min; (*d*) thereafter, 200 μl of sensitized sheep erythrocyte suspension is added which contains 4×10^8 EA in SVB and incubation at 20°C is continued for 15 min; (*e*) 200 μl of the reaction mixture is layered on 150 μl of a 40% sucrose solution in 0.05 M sodium phosphate buffer, pH 7, contained in a 400 μl polyethylene microfuge tube (Beckman Instruments, Inc., Clinical Instrument Division, Fullerton, Calif., cat. no. 314326). Centrifugation is performed at 15,000 rpm (approximately 13,000

g) for 5 min in a Beckman microfuge 152; (f) the microfuge tube is clamped with a hemostat 5 mm above the tip of the tube; (g) the tip of the microfuge tube containing the pelleted cells is cut off with a razor blade at the lower edge of the hemostat and is collected in a disposable tube. The upper portion of the microfuge tube including its contents is added to a second tube by releasing the hemostat; (h) the radioactivity in both tubes is measured; (i) the [^{125}I]C1q uptake by EA is calculated in percent of total radioactivity recovered:

$$\frac{\text{cpm in pellet}}{\text{cpm in pellet} + \text{cpm in supernate}} \times 100;$$

(j) to determine inhibition of [^{125}I]C1q binding to EA by the test sample, uptake of C1q by EA in presence of normal human serum is quantitated simultaneously. The percent inhibition of uptake (or the percent deviation of C1q by the test sample) is calculated:

$$\frac{\%\text{ uptake in control} - \%\text{ uptake in test}}{\%\text{ uptake in control}} \times 100.$$

The steps are summarized in Table I.

TABLE I
Procedure of the C1q Deviation Test

Step	Procedure
(a)	Mixing of 50 μl serum + 100 μl saline + 100 μl SVB.
(b)	Heating of mixture at 56°C for 30 min.
(c)	Addition of 1 μg [^{125}I] C1q.
(d)	First incubation: 15 min, 20°C.
(e)	Addition of 4×10^8 EA.
(f)	Second incubation: 15 min, 20°C.
(g)	Separation of cells and fluid phase in 40% sucrose, 13,000 g, 5 min.
(h)	Determination of distribution of radiolabel.
(i)	Calculation of uptake: $\frac{\text{cpm pellet}}{\text{cpm pellet} + \text{cpm supernate}} \times 100.$
(j)	Calculation of inhibition: $\frac{\%\text{ uptake (control)} - \%\text{ uptake (test)}}{\%\text{ uptake (control)}} \times 100.$

Results

Binding of [^{125}I]C1q to Sensitized Sheep Erythrocytes. Fig. 1 shows the extent of uptake of C1q by EA as a function of the conductance of the buffer used for suspending the cells. Maximal uptake was obtained at 6–8 mmho/cm. For the C1q deviation test a conductance of 7 mmho/cm was selected. Nonsensitized cells (E) showed no uptake even at low conductance.

Fig. 2 summarizes a comparative analysis of hemolysis and C1q uptake as a function of the relative amounts and types of antibody used. It indicated that in the case of IgM-type antibody, considerably more antibody is required for optimal C1q uptake than for maximal hemolysis. In the case of IgG-type antibody the requirements for both reactions are similar. Since large quantities of antibody cause hemagglutination, IgG-type anti-E was selected in preference to IgM for the C1q deviation test. A sensitizing dose of antibody was chosen which did not cause agglutination but allowed approximately 50% uptake under standard conditions.

Fig. 3 shows a dose response curve for C1q uptake by EA. To avoid hemolysis

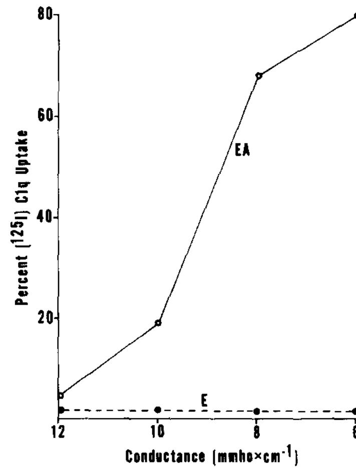

FIG. 1. Binding of [^{125}I]C1q to EA as a function of the conductance of the buffer.

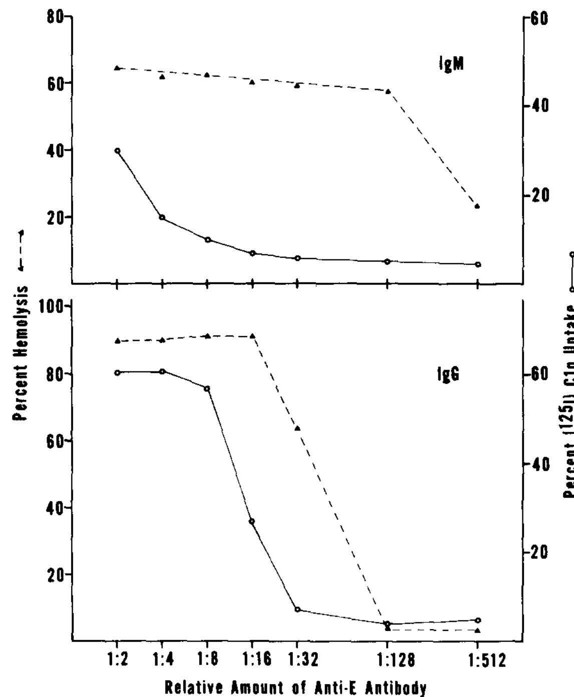

FIG. 2. Quantitative comparison of C1q uptake by EA and lysis of EA at different amounts of IgG or IgM antibody to sheep erythrocytes. Optimal sensitization for C1q uptake and hemolysis is similar for IgG-type antibody, but disparate for IgM type antibody.

the serum was either heated or fresh serum was used in the presence of EDTA (5×10^{-3} M). C1q uptake was somewhat greater in heated than in EDTA serum. Over a wide dose range, uptake was proportional to input. For the C1q deviation test, an amount of 1 μg of C1q was chosen, because this amount proved a sensitive indicator of small quantities of C1q-reactive material in the deviation test.

C1q uptake proceeds rapidly to 75% of the plateau value as depicted in Fig. 4. On the basis of this kinetic experiment 10 min at 20°C were chosen as the conditions for the C1q deviation test. Experiments not recorded here demonstrated that the temperature optimum for C1q binding was 20°C.

Although serum C1q does not significantly inhibit uptake of radiolabeled C1q (Fig. 3), addition to serum of unlabeled, isolated C1q produced a marked inhibitory effect (Fig. 5). This effect may indicate the specificity of [^{125}I]C1q binding.

Deviation of [^{125}I]C1q Binding to EA by C1q-Reactive Material Added to Human Serum. Increasing quantities of soluble aggregates of human IgG were added to various samples of a normal human serum and the mixtures were incubated for 60 min at 37°C. The samples were then submitted to the C1q

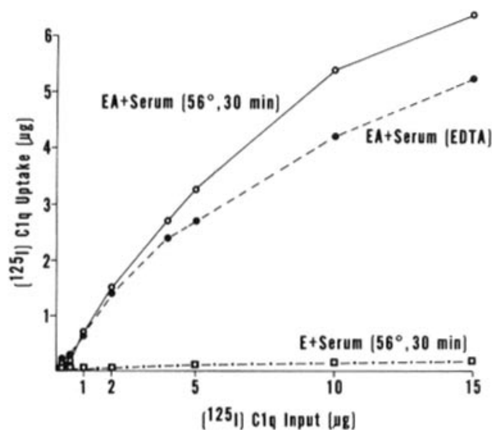

FIG. 3. [^{125}I]C1q uptake by EA as a function of input. Uptake was determined in presence of heated and unheated (EDTA 5×10^{-3} M) serum. As control served E.

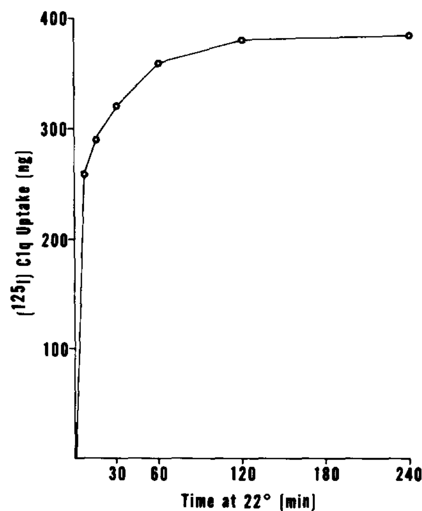

FIG. 4. Kinetic analysis of C1q uptake by EA. Uptake is 75% of maximal value at 10 min.

deviation test, both after heating at 56°C for 30 min and without prior heat treatment. Fig. 6 shows the sensitivity of the test in detecting IgG aggregates added to normal serum. Reproducibly, 5 μg of aggregates/ml of serum could clearly be detected, provided the test sample was heat-inactivated before testing. It is suggested that heat treatment frees the aggregates from bound complement

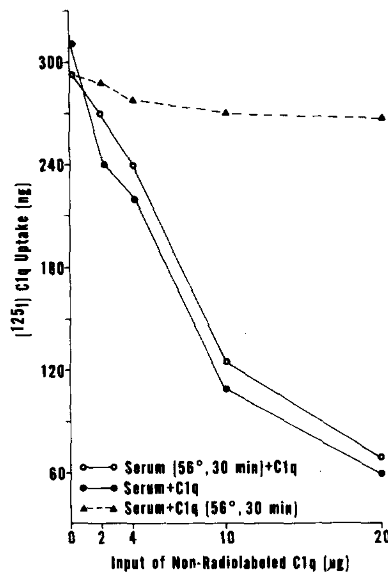

FIG. 5. Inhibition of uptake of radiolabeled C1q by nonradiolabeled C1q, and abolition of inhibition by heat treatment. All samples contained 1 μg [^{125}I]C1q and 5 $\times$ 10^{-3} M EDTA.

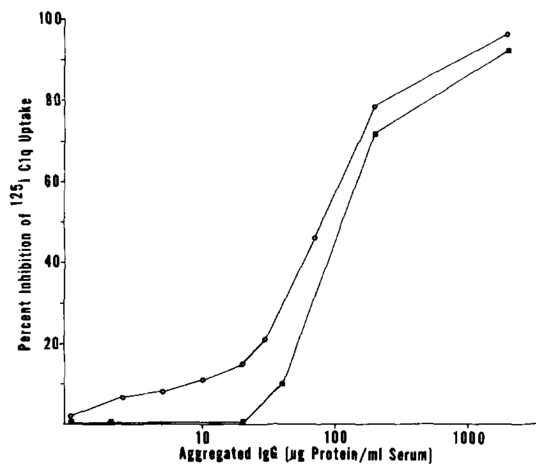

FIG. 6. Deviation of [^{125}I]C1q uptake by aggregated IgG. The indicated amounts of aggregates were first incubated for 60 min at 37° C in fresh human serum. The samples were then diluted 1:5 and one set of samples was heated for 30 min at 56° C in 5 $\times$ 10^{-3} M EDTA, whereas the other set received EDTA without heating. Detection of aggregates by the C1q deviation test is more sensitive after heating of the test samples.

proteins so that they become more reactive with C1q. The absolute amount of aggregates detectable in the volume of serum selected for the test is 250 ng. The value of C1q-reactive aggregates, however, is less than 100 ng because more than half of the material consisted of unaggregated IgG as determined by analytical ultracentrifugation.

To determine the reproducibility of the test, 10 different sera containing C1q-reactive material were subjected to replicate examinations. The coefficient of variation was 4.2%.

Fig. 7 demonstrates the capacity of the C1q deviation test to detect soluble immune complexes. After removal of the precipitates of BSA and rabbit anti-BSA, the supernates were tested for C1q-reactive material and for hemolytic complement consumption. The results of the C1q deviation test correlate with those obtained by quantitation of hemolytic complement consumption.

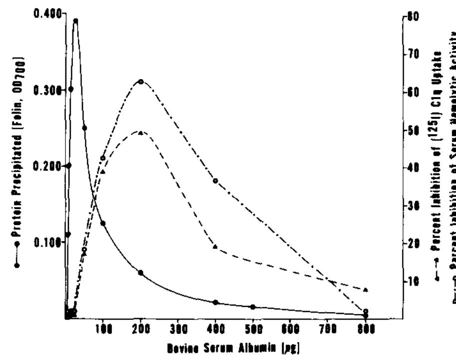

FIG. 7. Deviation of [^{125}I]C1q uptake by soluble immune complexes: correlation between C1q-reactive complexes and complexes capable of activating complement. The antigen was bovine serum albumin, the antiserum was of rabbit origin. After removal of the precipitates, identical aliquots of all supernates were analyzed.

Various bacterial and phage products were tested for their ability to activate complement. Using genetically C2-deficient serum and serum rendered deficient in C3 proactivator by heating at 50°C for 20 min, it was determined whether activation of complement proceeded via the classical or the alternative pathway or both. These results were compared with data obtained by analysis of the same products with the C1q deviation test and the C1q precipitation-in-gel test (1). The results are summarized in Table II and show that by and large substances which activate the classical complement pathway are detected by the C1q deviation test. They also reveal a good qualitative correlation between the results obtained with the latter test and the C1q precipitation-in-gel test. Highly reactive bacterial products could readily be detected in the C1q deviation test at concentrations of 20–30 μg per ml of serum.

C1q reacts with single- and double-stranded DNA as has been demonstrated by Agnello et al. (7). An examination of the sensitivity of the C1q deviation test for the detection of DNA in serum, showed that as little as 5 μg DNA/ml serum is detectable, or 250 ng per test sample (50 μl). The dose response curves for native and denatured DNA and the abolition of the reaction by DNAse are demonstrated in Fig. 8.

TABLE II

Detection of Bacterial and Phage Products in Serum by the C1q Deviation Test: Comparison with C1q Precipitation-In-Gel Test and Correlation with Consumption of Complement by the Classical or Alternative Pathway

Products of:*	Inhibition of [125I]C1q uptake‡	C1q Precipitation§	Complement consumption via‖	
			C1, 4, 2	Properdin
	%			
E. coli 08	47	++	++	0
E. coli 0136	73	++	++	0
E. coli 0115	37	++	++	0
Staphylococcus G7	22	+	++	0
Staphylococcus Copenhagen	0	0	+	+++
Staphylococcus phage 80	94	++	+	++
Pneumococcus R3	18	++	+++	0
Streptococcus L C.W.	0	0	0	0
S. typhosa 0901	12	0	0	++
B. subtilis	0	0	0	+++

* For detailed description see Materials and Methods.
‡ 350 μg.
§ 3.5 mg/ml.
‖ 700 μg/ml.

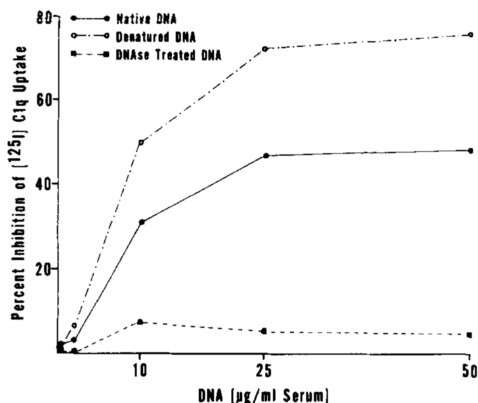

FIG. 8. Deviation of [125I]C1q uptake by native, denatured and DNAse treated DNA.

Application of the C1q Deviation Test to the Analysis of Pathological Sera. 193 samples of serum from 43 patients with Dengue hemorrhagic fever were analyzed. The results were correlated with the severity grade of the disease in each case. As shown in Fig. 9 the least inhibition of [125I]C1q uptake was seen in group I which comprises patients with mild disease. The highest degree of inhibition was associated with the occurrence of severe shock which is characteristic for patients of group IV. When serial samples of individuals with disease grade III and IV were tested, the most positive C1q deviation tests were observed in serum obtained on the day of shock.

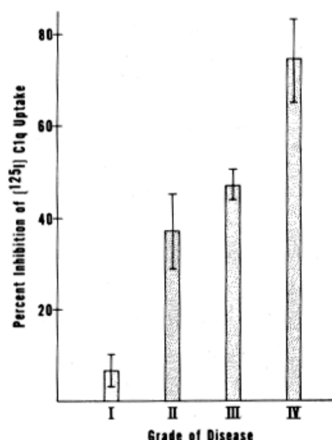

FIG. 9. Application of the C1q deviation test to an analysis of serum samples from patients with Dengue hemorrhagic fever. A total of 193 samples were analyzed. The bars represent the standard error of the mean.

Discussion

Several different approaches have been employed in search for a method capable of detecting soluble immune complexes in serum and other body fluids. These approaches included the utilization of hemolytic complement consumption (8), C1q precipitation (1, 9), precipitation by monoclonal rheumatoid factor (2), binding to cellular complement receptors (10), or Fc receptors (11), and inhibition of phagocytosis (12).

It was the purpose of this study to develop a simple, rapid, and economical test with a high degree of sensitivity for complement reactive substances. C1q was employed because, being the recognition protein of the classical complement system, it has proved to be a sensitive detector of immune complexes and other complement reactive materials. To achieve maximal sensitivity, binding of C1q to immune complexes in serum was not measured directly. Instead, we quantitated the interference of these complexes with C1q binding to sensitized sheep erythrocytes (EA). In a mixture of EA, soluble immune complexes (AGAB) and C1q, a proportion of C1q is deviated and bound by the complexes. This amount, which is related to the quantity and reactivity of the complexes present, is prevented from binding to EA. Compared to a complex-free reaction mixture, EA-bound C1q is thereby diminished. The reactions of the control (*a*) and the test (*b*) mixtures may be represented as (*a*) EA + C1q → EAC1q + C1q; (*b*) EA + AGAB + C1q → EAC1q + AGABC1q + C1q. C1q was detected by a radiolabel after separation of cells and fluid phase. Although the introduction of ^{125}I by the chloramine T method may abolish C1q hemolytic activity, it does not impair the immunoglobulin-binding activity of the protein (13). Separation of cell-bound and fluid phase C1q was accomplished by layering the mixture on a high density sucrose solution which was followed by brief centrifugation. After centrifugation, complex-bound and free C1q (top) are separated from EAC1q (bottom) by a zone which was completely devoid of radioactivity. Quantitation of radioactivity in

both fractions allows accurate determination of C1q deviation from EA by C1q-reactive substances. For optimal sensitivity, the amount of hemolysin used to prepare EA is critical. Since individual batches of hemolysin differ in C1q reactivity, the optimal sensitizing dose with respect to C1q binding has to be determined for each batch. This dose may differ considerably from the optimal sensitizing dose used for complement-dependent hemolysis.

The sensitivity of the C1q deviation test is significantly greater than that of other tests utilizing C1q for the detection of soluble immune complexes. Whereas the C1q precipitation-in-gel test and the radiolabeled C1q precipitation test detect γ-globulin aggregates at minimum concentrations of 100 μg/ml and 50 μg/ml, respectively, C1q deviation allows the detection of 5 μg/ml. The smallest absolute amount of aggregates detectable by C1q deviation is approximately 250 ng. It is also noteworthy that the entire test can be completed within 1–2 h, and that 50 μl of test serum is sufficient for one determination. The use of glutaraldehyde-fixed EA in the test is presently under investigation. Given the stability of such modified indicator cells and the known stability of radiolabeled C1q on storage over longer periods of time, the routine performance of the test may become feasible in clinical laboratories. It is probable that the sensitivity of the test can be further enhanced by perfecting the methodology.

The C1q deviation test is similar in sensitivity to the test employing cultured lymphoblastoid cells for the binding of immune complexes (10). However, the two reactions are governed by different principles. Lymphoblastoid cells bind immune complexes present in serum primarily by virtue of their complement receptors for C3b and C3d which react with the complex-bound complement proteins. The reaction of the complexes in serum with complement thus is a prerequisite for their detectability by lymphoblastoid cells. In contrast, C1q reacts directly with the immunoglobulin of immune complexes provided that it is of the IgG or IgM type. Complement bound to the immune complexes may therefore reduce their reactivity with C1q. Apparently, heating at 56°C reverses binding of complement to these complexes and renders them accessible to C1q. The reactivity of IgG aggregates previously incubated in fresh serum was increased after heat treatment of the mixture (Fig. 6). In order to minimize or to prevent the aggregation of serum γ-globulin during heat treatment, the test serum was diluted fivefold before heating. There was no evidence that this treatment resulted in an increase of C1q-reactive material. However, the possibility exists that C1q reactants which are relatively thermolabile, may be inactivated.

In the test developed by Nydegger et al. (9) it is necessary to separate free C1q from C1q which is bound to soluble immune complexes. This is accomplished by differential precipitation of the latter at a critical concentration of polyethylene glycol. Separation of two soluble forms of C1q is not required in the C1q deviation test. Instead, free and complex-bound C1q are removed together and separated from solid-phase C1q (EAC1q) by the simple and highly accurate technical procedure described. Mere washing of EAC1q before quantitation of cell-bound C1q was not feasible because of the reversibility of C1q binding. Washing also renders quantitation of fluid-phase C1q inaccurate or impossible.

C1q is known not only to react with aggregated γ-globulin and immune

complexes, but also with certain endotoxins (14) and with DNA (15). These non-Ig substances are also recognized in the C1q deviation test, which must be borne in mind in regard to the evaluation of a positive result.

A limited and preliminary application of the test to clinical material has been performed. Patients with Dengue hemorrhagic fever may be categorized according to severity of disease in grades I–IV. Sera from patients with disease grade IV exhibited the highest degree of C1q deviation and those from patients with grade I the lowest. Serial serum samples of individual grade IV patients gave the highest value for C1q deviation at the day of shock when the serum concentration of C3 and other complement proteins was lowest compared to earlier or later serum samples. The nature of the C1q-reactive material in Dengue serum has not been determined. However, the partially purified material was able to activate complement in normal human serum. For the isolation of C1q-reactive material from Dengue and from other pathological sera, the deviation test should prove a useful assay for tracing the material through consecutive preparative steps.

Summary

This report describes a new, rapid, sensitive, and quantitative method for the detection of immune complexes, endotoxins, and other complement activating materials in patient's sera utilizing the ability of these substances to react with isolated C1q. The procedure is based on the inhibition of radiolabeled C1q binding to sensitized sheep erythrocytes by C1q-reactive substances in pathological sera. The C1q deviation test may be performed on 50 μl of serum, using 1 μg of radiolabeled C1q per sample. The procedure may be completed in 1.5–2 h, it is capable of detecting 5 μg of aggregated human IgG per ml of serum, and its coefficient of variation is 4.2%. Application of the test to the study of 193 sera from 43 patients with Dengue hemorrhagic fever showed a positive correlation between degree of C1q deviation and severity of disease.

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