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A C1q SOLID PHASE MICROENZYMATIC ASSAY FOR THE DETECTION OF SOLUBLE IMMUNE COMPLEXES °

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Clinical and serological evidences indicate that circulating immune complexes (CIC) play a role in pathophysiologic phenomena occurring in humans and in various spontaneous and experimentally induced animal diseases[1]. Because in several instances the presence of CIC correlates significantly with the clinical activity of the disease, a number of assays have recently been developed to detect CIC in serum and other biological fluids[12,16]. As observed in a collaborative study[5], each assay suffers from methodological problems such as low or variable sensitivity in different diseases, difficulties in performance and adaptation to mass screening. This has stimulated a growing interest in developing easy to perform but still sensitive techniques with a view to test a large number of samples for CIC in more than one assay.

In the present investigation the solid phase C1q-binding assay[3,10] has been modified into a microenzymatic method employing alkaline phosphatase labeled soluble protein A.

MATERIALS AND METHODS

Antigens and antisera

Human C1q was purified by the modification of the method of YONEMASU and STROUD[15], described by ZUBLER et al.[17]. All the preparations were tested for purity by

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double diffusion in agarose against a horse anti-human whole serum (*Behring Institute*) and rabbit anti-human IgG, IgM, IgA antisera (*Behring Institute*).

Bovine serum albumin (BSA), employed throughout this study, was also purchased from *Behring Institute*. Antisera anti-C1q and anti-BSA were obtained by immunization of New Zealand albino rabbits. Animals received four i.m. injections of 0.04 mg of purified C1q and 20 mg of BSA, respectively, in complete Freund adjuvant once a week for four weeks.

Human IgG were aggregated at 12 mg/ml of saline by incubation for 20 min at 63 °C. Insoluble aggregates were removed by centrifugation at 3,000 *g* for 5 min. The concentration of the stock solution was then adjusted to 10 mg/ml, reading the optical density at 280 nm (OD_{280}) in a Zeiss spectrophotometer. Heat-aggregated human IgG (Agg-IgG) were stored in small aliquots at −70 °C and thawed only once.

C1q-coated plates

Disposable polystyrene or polyvinyl microtiter plates with 96 round bottom wells were both employed with satisfactory results.

Wells were coated with C1q as follows: 0.2 ml of C1q solution (0.01 mg/ml) in phosphate (0.015 M) buffered saline (0.15 M), pH 7.2 (PBS), containing 0.02% sodium azide, added to single wells. After 48 h of incubation at 4 °C, the plate was washed three times with 0.2 ml of cold PBS containing 0.05% tween 20 (PBS-tween). The unreacted protein-binding sites of the wells were saturated by adding 0.2 ml of PBS containing 0.3% of gelatin and incubating the plate at room temperature for 2 h. After additional three washes with PBS-tween, the plate was stored at −70 °C or a few days at 4 °C after having filled wells with PBS azide. To verify the coating of the plate with C1q, 0.2 ml of rabbit anti-C1q antiserum and, as control, an equal amount of normal rabbit serum, were added to the plate in triplicate and the binding was detected with enzyme labeled protein A, as described in the assay.

Alkaline phosphatase conjugation to staphylococcal protein A

Soluble staphylococcal protein A (SPA) was labeled with alkaline phosphatase Sigma type VII, specific activity 1,200 U/mg, using glutaraldehyde and following the method described by ENGVALL and PERLMANN[2]. Unlinked proteins were removed from the conjugate by gel-filtration through a column (1.6 x 70 cm) of Sephadex G-100 in 0.1 M tris-HCl, pH 8. The first eluted peak containing enzyme labeled SPA was collected and stored at 4 °C after dilution to 10 ml with buffer containing 0.02% gelatin, $MgCl_2$ 0.001 M and 0.02% sodium azide.

Titration of the SPA-alkaline phosphatase (SPA-AP) conjugate

Titration of the activity of the conjugate was performed to establish the optimal dilution to be used in the immune complex assay. Briefly, microtiter wells were coated with human IgG (0.01 mg/ml) in carbonate buffer (0.05 M), pH 9.6, as described for C1q-coating. Wells were then incubated with 0.2 ml of rabbit anti-human IgG antiserum and diluted in PBS-tween to 0.01 mg/ml of gammaglobulin content. The plate was then incubated at 37 °C for 1 h; wells were thereafter washed with PBS-tween and incubated with 0.2 ml of twofold dilutions of the SPA-AP conjugate in PBS-tween. The amount of conjugate bound to the wells was estimated as described below. The highest dilution of conjugate giving the same amount of hydrolyzed substrate produced by the undiluted preparation was chosen as working dilution in the immune complex assay.

In vitro preformed BSA-anti-BSA soluble immune complexes

Quantitative precipitin curves were obtained for the rabbit BSA-anti-BSA IC, soluble immune complexes (sIC) from two to ten times antigen excess were prepared by incubating equal volumes of antiserum (0.25 ml) and antigen solution for 1 h at 37 °C and 48 h at 4 °C. At the end of the incubation, the insoluble material was removed by centrifugation (7,000 *g* for 20 min) and the supernatant used in the IC assay.

Animal sera

Sera from rabbits with chronic serum sickness (CSS), induced by injection of BSA, were collected 15 min after the daily injection of the antigen. The amount of BSA inoculated into these animals was calculated on the basis of their anti-BSA antibody titer[14].

Human sera

Sera examined for the presence of CIC came from 10 apparently normal donors, from 25 patients with systemic lupus erythematosus (SLE) in different forms of clinical activity, from 18 patients with malignant melanoma of the primary and recurrent types, from 10 patients with seropositive rheumatoid arthritis, from 7 patients with chronic active hepatitis and 12 with essential mixed cryoglobulinemia (EMC). Some of the above mentioned sera were kindly provided by the *Servizio di Reumatologia, III Cattedra di Patologia Medica, Università di Pisa* and by *Ospedale Fatebenefratelli, Roma.*

C1q solid phase (C1q-SP) enzyme microassay

All samples to be tested were prepared as described in a previous article[7]. Briefly, each sample was diluted 1:3 in Na_2EDTA (0.2 M), pH 7.2, containing 0.05% tween 20. Positive standards consisted of one volume of Agg-IgG (concentration ranging from 0.015 to 3 mg/ml in saline), one volume of normal human serum (NHS) (30 min at 56 °C), one volume of Na_2EDTA. Before the test, EDTA-containing samples were incubated at 37 °C for 30 min and then transferred to an ice-bath.

The assay was performed in triplicate, as follows: 0.150 ml PBS-tween and 0.050 ml of sample were added to the C1q-coated wells. Six wells were filled with 0.2 ml of PBS-tween and were used to control the aspecific binding of protein A to C1q and the aspecific hydrolysis of substrate by C1q.

The plate was incubated for 1 h at 37 °C, followed by 30 min at 4 °C. After repeated washings, 0.2 ml of SPA-AP, appropriately diluted with PBS-tween (average 0.025 mg/ml), were added to each well and the plate incubated and washed as above.

The SPA-AP bound was determined by adding 0.2 ml of p-nitrophenylphosphate in 0.05 M carbonate buffer, pH 9.6, with 0.001 M $MgCl_2$. The plate was allowed to stand 30 min at room temperature and the enzymatic reaction stopped by adding to each well 0.05 ml of 2 N NaOH.

The amount of hydrolysis was determined by reading with a Zeiss spectrophotometer the optical density at 400 nm (OD_{400}) of the yellow colour of the released p-nitrophenolate.

RESULTS

Sensitivity of the C1q-SP-enzyme microassay for detection of CIC

The sensitivity of the microassay was established by measuring the C1q-binding activity of Agg-IgG. To this purpose, different concentrations of Agg-IgG were added to heat-inactivated NHS and the solution tested in the enzyme micromethod. Figure 1 shows the results of four experiments and demonstrates that as little as 0.5 μg (0.030 mg/ml) Agg-IgG can be measured by the microassay.

Characterization of sIC detected by the C1q-SP enzyme microassay

To establish the molecular size of IC measured by the present method, rabbit BSA-anti-BSA sIC were prepared *in vitro* using different antigen to antibody ratios and tested for binding in the microenzymatic test. Figure 2 demonstrates that sIC formed at 3, 6 and 10 times antigen excess were all detected. While the micromethod seems to measure preferentially sIC formed in slight antigen excess, a significant binding can still be appreciated with sIC of smaller size, such as those prepared with amounts of antigen that are 10 times greater than that needed for equivalence.

Detection of sIC in animal sera

Sera of rabbits with chronic serum sickness (CSS) produced by daily injections of BSA were obtained from animals prior and after the daily antigen i.v. injection and analyzed in their sIC content. Table 1 summarizes the results of this experiment. While normal rabbit serum and sera of CSS rabbits before the BSA administration were negative in the microassay (0.3-0.34 OD_{400}), the two rabbit sera collected after the antigen i.v. injection displayed a comparable concentration of IC (0.940-1.0 OD_{400}).

Detection of IC in patient sera

Before attempting to measure the CIC in human sera, the range of non-specific binding of NHS was established using sera from ten apparently normal subjects. The mean binding value was 0.385 ± 0.155 standard deviation (SD) OD_{400}. Thus, a pathological serum was defined as CIC positive when giving a binding of 0.850 OD_{400}, equal to 3 SD over the mean value of NHS. Table 2 reports the results of the screening of unselected pathological human sera obtained from patients with different diseases in the C1q-SP-enzyme microassay. High percentages of CIC positive sera were found among patients with SLE (52%) and patients with chronic active hepatitis (57%). Lower percentages of positive values were found in sera of patients with malignant melanoma (28%), rheumatoid arthritis (30%) and EMC (17%). Positive values ranged between 0.850 and 2.0 OD_{400}.

DISCUSSION

In the present study the C1q-solid phase binding test has been adapted to the enzyme microassay using enzyme labeled soluble staphylococcal protein A in place of the second antibody. The SPA-AP conjugate is easy to prepare and provides a very stable reagent over a period of months, which avoids the preparation of specific anti-Ig purified antibodies. Furthermore, the micromethod allows the testing of a large number of samples and it can be employed in animal studies in which IC bind C1q and the Fc fragment of antibody has an affinity for SPA[9]. In this context, it

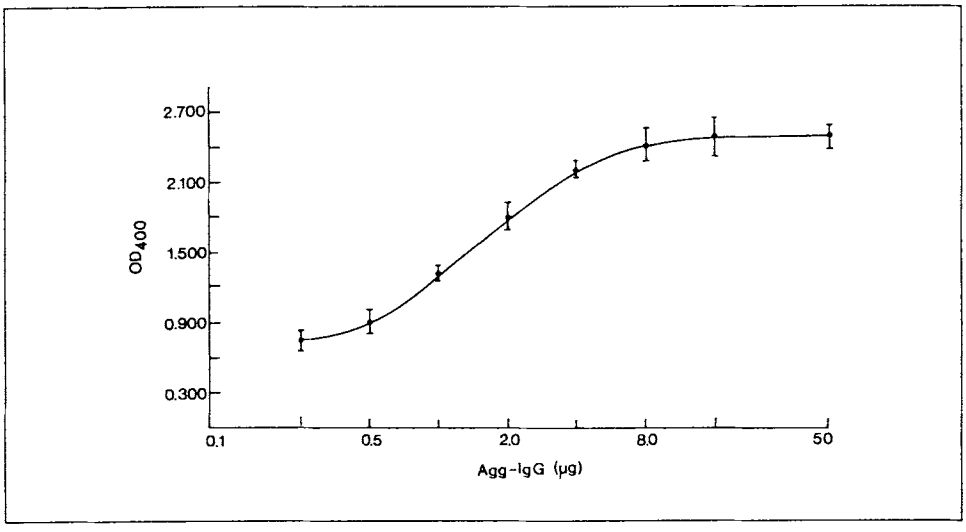

Fig. 1 - Sensitivity of the C1q-SP-enzyme microassay. On the abscissa, µg of Agg-IgG added to each well at the concentrations of 0.015, 0.030, 0.060, 0.125, 0.250, 0.500, 1 and 3 mg/ml are reported. The OD_{400} corresponding to the amount of hydrolyzed substrate are shown on the ordinate. Mean values of four experiments and the range between maximum and minimum value are plotted.

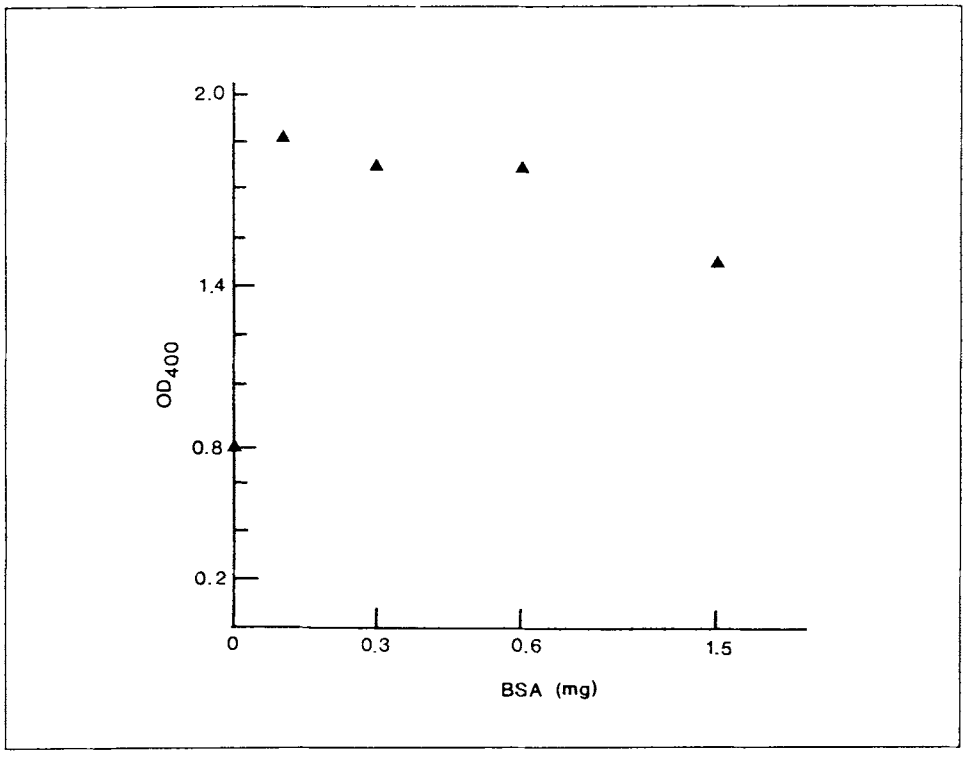

Fig. 2 - Values of hydrolysis obtained in the C1q-SP-enzyme microassay by the binding of BSA-anti-BSA sIC prepared at the equivalence (0.1 mg of antigen), 3 (0.3 mg), 6 (0.6 mg) and 10 times (1.5 mg) antigen excess.

animals	before antigen injection (OD_{400})	after antigen injection (OD_{400})
rabbit 1	0.300	0.940
rabbit 2	0.340	1.000

Tab. 1 - Presence of circulating immune complexes (CIC) in sera of rabbits with chronic serum sickness.

clinical diagnosis	no. of cases	no. of positive cases (%)
malignant melanoma	18	5 (28)
systemic lupus erythematosus	25	13 (52)
rheumatoid arthritis	10	3 (30)
chronic active hepatitis	7	4 (57)
essential mixed cryoglobulinemia	12	2 (17)

Values of binding greater than 0.850 OD_{400} equal to three SD over the mean value of 10 human control sera (0.385 ± 0.155 OD_{400}).

Tab. 2 - Presence of soluble immune complexes (sIC) in the pathological sera.

should be remembered that a limitation of the present test is the variable affinity for SPA of different classes and subclasses of antibodies. Although not explored in the present study, this limitation could be overcome by a sandwich technique where prior incubation of the plate with SPA would be preceded by the reaction of the wells with a rabbit anti-Ig antiserum, which avidly binds SPA.

The sensitivity of the C1q-SP-enzyme microassay is comparable to that of conventional C1q-binding test and 0.030 mg/ml of Agg-Ig can be measured. The test is highly reproducible and it can be performed on C1q-coated plates, which are stored dry one month[4] at −70 °C and for shorter time periods at 4 °C, provided that the reaction wells are filled with PBS-azide.

The method appears to be capable of detecting sIC formed on a wide range of antigen-antibody ratio. Thus, sIC prepared *in vitro* at ten times antigen excess can still be measured. To assess the clinical application of the described method, we tested a number of sera from patients with different pathological conditions known to be associated with CIC.

IC associated with melanoma has been detected by the Raji cells assay[11,13] and it was found that malignant melanoma patients with active disease had higher levels of IC than those with no clinical evidence of the disease. Our group of melanoma patients did not receive any treatment at the time of testing; 9 out of 14 had metastatic disease and in 4 of them measurable concentration of CIC in serum could be found.

IC-like material was frequently detected in sera of SLE patients by almost every currently available technique. A WHO collaborative study[5] confirmed this finding, showing that six different methods, including C1q-SP, could discriminate between controls and SLE sera. We found 52% of positive sera from a group of SLE patients randomly selected and with various degrees of clinical activity of the disease.

Results obtained among a group of patients with chronic hepatitis seem to confirm previous reports[8], although the number of cases studied here is too low to draw significant conclusions.

Different techniques have been used to measure IC in sera and synovial fluids of patients with rheumatoid arthritis and several degrees of positivity for IC-like materials were found, as reviewed by THEOFILOPOULOS and DIXON[12]. The number of positive cases that we were able to detect was very low, confirming the low sensitivity of the C1q-SP for this kind of disease[5].

LAWLEY et al.[6] have recently demonstrated that sera of EMC patients contain large amounts of sIC that could be detected by the C1q-binding assay. With C1q-SP-enzyme microassay we could not confirm these data. This discrepancy might be due to the fact that our patients are randomly sampled and different activities of the disease might be responsible for the results.

SUMMARY

The solid phase C1q-binding assay has been adapted to an enzymatic micromethod in which alkaline phosphatase labeled soluble *Staphylococcus aureus* protein A is used in place of the second antibody. The assay, which is run in microtiter plates, provides a rapid, sensitive (0.030 mg/ml of human heat-aggregated IgG detected) and reproducible method for the measurement of soluble immune complexes in a large number of samples. Soluble immune complexes prepared *in vitro* with bovine serum albumin (BSA) and anti-BSA antibodies on a wide range of antigen to antibody ratios were all detected with this method. When applied to the screening of unselected patient sera, soluble immune complexes were frequently found in systemic lupus erythematosus (52%) and chronic active hepatitis (57%) and in lower percentages in patients with malignant melanoma (28%), rheumatoid arthritis (30%) and essential mixed cryoglobulinemia (17%).

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