Circulating Immune Complex-Like Materials Which Bind to Heat Inactivated Clq Interfere with the Clq Solid Phase Assay for Immune Complexes

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FUKUDA, K., SEINO, J., KINOSHITA, Y., SUDO, K., HORIGOME, I., SAITO, T., FURUYAMA, T. and YOSHINAGA, K. Circulating Immune Complex-Like Materials Which Bind to Heat Inactivated Clq Interfere with the Clq Solid Phase Assay for Immune Complexes. Tohoku J. exp. Med., 1985, 146 (4), 449-456, Clq solid phase assay (Clq-SP) was devised based on the fact that immune complexes (IC) and aggregated human globulin (AHG) bind to Clq. Neither IC nor AHG was found to bind to heat inactivated Clq. On the other hand, circulating immune complex (CIC)-like materials in patients' sera were able to bind to heat inactivated Clq, indicating that these CIC-like materials are not true CIC. Gel filtration analysis showed that molecular size of such CIC-like materials was almost the same as monomeric IgG, while true CIC were in heavy fractions. True CIC did not bind to heat inactivated Clq but bind only to native Clq. The CIC-like activity is not due to rheumatoid factors. About 2/3 of CIC positive sera by Clq-SP are not really CIC positive but are due to interference by the CIC-like materials.

In lupus nephritis, membranous nephritis, IgA nephritis and other forms of glomerulonephritis, deposits of immune complexes (IC) have been demonstrated at the sites of the glomerular lesion, and the IC were thought to give rise to nephritis by activating complement in the renal tissue.

Recently, measurement of circulating immune complexes (CIC) were attempted in order to elucidate the mechanism of the nephritis and to use them as a guide of therapy. However, biological activity of IC is so intricate that a great variety of CIC assays have been tried. Among these assays, Clq solid phase assay (Clq-SP) (Hay et al. 1976) is generally thought to be the most reliable.
Clq-SP, serum samples were incubated with solid phase Clq. After washing, $^{125}$I-anti-IgG or anti-IgG-peroxidase was added and incubated. After washing, the amount of IgG bound to solid phase Clq was interpreted as the CIC value. However CIC-like activity detected by Clq-SP was often found in 7S fractions or IgG fractions in sucrose density gradient analysis or gel filtration analysis (Theofilopoulous et al. 1976; Tung et al. 1981; Uwatoko et al. 1984a; Hack et al. 1984; Marder et al. 1984). Whether this 7S CIC-like IgG contains a small molecule in its antigen-binding site or has activity to bind Clq independently of Clq activity is not known. We investigated this aspect using heat inactivated Clq on solid phase.

**Materials and Methods**

**Sera of patients.** Blood samples were collected from patients with chronic glomerulonephritis and those with systemic lupus erythematosus (SLE). Blood was kept at room temperature for 1 hr and centrifuged at 3,000 × g for 15 min. Serum samples were stored at −80°C and examined within 4 weeks. Once thawed, the sera were not stored again.

**Purification of Clq.** Rabbit and human Clq were obtained according to the method of Volanakis and Stroud (1972). The Clq was further purified by Sephadex G-200 gel column in 0.65 M NaCl containing 10 mM EDTA and 10 mM sodium phosphate, pH 7.5 (Kolb et al. 1979). The fractions containing Clq activity were applied to gelatin-Sepharose to remove contaminating fibronectin (Kenneth et al. 1983). The purity of Clq was confirmed by 10% polyacrylamide gel electrophoresis containing 0.1% sodium dodecyl sulfate (SDS-PAGE).

**Clq solid phase enzyme immunoassay.** 100 μl of Clq (10 μg/ml) was added to each well of a polystyrene microtiter plate and kept at 4°C overnight. After washing the well with phosphate buffer saline (PBS)-0.05% Tween 20, 200 μl of bovine serum albumin (BSA) in PBS was added and incubated at 37°C for 1 hr to saturate uncoated well. Then, the well was washed with PBS-Tween 20 again, and 100 μl of serum sample diluted 1:12 with dilution buffer (PBS containing 0.05% Tween 20, 0.5% BSA and 10 mM EDTA) was added and incubated at 37°C for 1 hr. After washing the well with PBS-Tween 20, anti-IgG labeled with horseradish peroxidase (HRPO : Sigma Co.) was added and incubated at 37°C for 1 hr, and the bound HRPO activity developed with o-phenylenediamine was determined by optical density scanning of the plate at OD$_{492}$ (Corona E.Co., MTT-12). In order to investigate the CIC-like activity, Clq (10 μg/ml) was incubated at 0°C, 37°C, 45°C, 50°C, 53°C, 56°C and 60°C for 15 min, and was used for Clq SP.

**Gel filtration analysis.** Serum samples in a volume of 0.5 ml were applied to a column of Sephadex G-200 gel equilibrated with PBS containing 2 mM EDTA, and 3 ml fractions were collected and analysed by Clq-SP.

**Pepsin digestion of CIC-like IgG.** Three samples exhibiting high CIC-like activity were dialysed against 0.01 M phosphate buffer containing 2 mM EDTA, pH 8.0 and applied to DEAE Sepharose columns equilibrated with the same buffer. The concentrations of obtained IgG fractions were adjusted to 1 mg/ml, and digested by 1/30 pepsin in 0.05 M acetate buffer, pH 4.45. The products of pepsin digestion were applied to a Sephadex G-100 gel column and the first peaks were applied to a protein A-Sepharose column to remove intact IgG. The CIC-like activities of IgG and F (ab')$_2$ fragments were analysed by Clq-SP using heat inactivated by human Clq.
**RESULTS**

As incubation of Clq at the temperatures over 53°C for 15 min results in inactivation of Clq, either AHG or HRPO-anti-HRPO immune complex (Px-anti-Px) did not bind to such heat inactivated rabbit Clq (Fig. 1, a). However, CIC-like materials in patient’s sera were found to still bind to the heat inactivated rabbit Clq (Fig. 1, b) though some decreases in binding were seen. Incubation of Clq at 60°C for 15 min did not alter the antigenicity of Clq (Fig. 1, a).

Gel filtration analysis showed that there were two major peaks of CIC-like activity. One peak was in the void volume (IgM fractions) and the other in IgG fractions. The CIC-like materials in IgG fractions were able to bind to the heat inactivated Clq, while CIC-like materials in the void volume did not (Fig. 2). These data indicate that CIC-like materials in heavy fractions are probably true CIC, but CIC-like materials in IgG fractions are not. The CIC-like activity was not due to IgG rheumatoid factors, because CIC-like activity was not correlated with IgG rheumatoid factors (Fig. 3).

Interference with the assay by such CIC-like materials was also seen when human Clq was used for Clq-SP. Fig. 4 shows the binding properties of CIC-like materials to human Clq. Forty serum samples indicating high CIC levels by conventional Clq-SP using human Clq were tested. CIC-like materials were

![Figure 1](image)

**Fig. 1.** Rabbit Clq (10 μg/ml in PBS) was incubated at each temperature for 15 min and used for Clq-SP.

a: Binding properties of AHG at each concentrations (solid line) and HRPO-anti-HRPO complex (Px-anti-Px: dotted line) and anti-Clq (dotted line) to heated Clq.

b: Binding properties of CIC-like materials in patients’ sera to heated Clq. These 13 samples were CIC positive by conventional Clq-SP.
Fig. 2. Sephadex G-200 gel chromatography of serum samples: Each fraction was analysed by C1q-SP with native rabbit C1q (solid line) and with heat inactivated rabbit C1q (dotted line). $V_0$: void volume.

Fig. 3. Relation between IgG rheumatoid factors (RF) reacting with rabbit IgG and CIC-like materials reacting with heat inactivated rabbit C1q.
found to still bind to the heat inactivated human Clq in more than half cases. When the differences of absorbance values between CIC activities reacting with native Clq and CIC-like activities reacting with heat inactivated Clq were calculated as true CIC, 26 samples (2/3) were CIC negative and only 14 samples (1/3) were positive. The normal range was set by the values of sera from 10 apparently healthy persons.

In order to investigate whether the activity of CIC-like materials was pepsin sensitive or not, IgG and F (ab')₂ fractions were obtained from three serum samples as described in materials and methods. The IgG thus prepared was found to bind to the heat inactivated human Clq in every case, and the F (ab')₂ fraction still had the binding activity though significantly weakened (Fig. 5).

**DISCUSSION**

Among the large numbers of CIC assays Clq-SP is widely used as the most reliable one. IC and AHG are able to bind to Clq while monomeric IgG is not. If CIC bind to Clq, it is reasonable to assume that CIC must bind endogenous Clq and molecular weights of such CIC-Clq complexes must be more than 560 kd. But, CIC-like activity by Clq-SP is often found in 7S fractions or IgG fractions. These data suggest that CIC-like materials do not bind to endogenous fluid phase
native Clq but bind only to the solid phase Clq (Uwatoko et al. 1984b). Hack et al. (1984) also reported that CIC detected by Clq-SP did not activate serum complement and bear neither C4 nor C3.

No one knows if the CIC measured by Clq-SP are true CIC or not. We used heat inactivated rabbit and human Clq for Clq-SP and revealed that at least 2/3 of CIC detected by conventional Clq-SP were not true CIC but IgG which was able to bind to solid phase or aggregated Clq independent of Clq activity. Such binding mode has been observed between Clq and fibronectin (Kenneth et al. 1983). Fibronectin does not bind fluid phase native Clq but binds to solid phase Clq or heat aggregated Clq or gelatin. But the CIC-like activity by Clq-SP is not due to the fibronectin, because the CIC-like materials did not bind to the solid phase gelatin or fibronectin (data not shown).

We attempted to purify this CIC-like IgG by heat inactivated human Clq-Sepharose 4B affinity column, but failed probably because of interference by endogenous Clq which aggregated during the procedure. Marder et al. (1984) were able to purify the 7S CIC-like IgG (called Clq precipitins by Agnello et al. 1971) from the sera of patients with hypocomplementemic vasculitis urticaria syndrome (HVUS) by Clq-coated polystyrene beads. HVUS is characterized by the presence of circulating 7S protein (IgG) that precipitates Clq in an agarose double diffusion gel of low ionic strength, and also by a profound decrease in serum Clq, rendering the affinity purification possible. As we failed to purify the CIC-like IgG by affinity chromatography, we purified it by ion exchange chromatography and digested it by pepsin. CIC-like materials are sensitive to pepsin digestion. Marder et al. (1978) also reported that Clq precipitins are

Fig. 5. Binding of CIC-like IgG (solid line) and its F (ab')2 fragments (dotted line) to heat inactivated human Clq on solid phase. Closed circle, open circle and quadrangle indicate individual samples.
pepsin sensitive, though the exact mechanism of the interaction between Clq and CIC-like materials is not known.

Human Clq is generally used for Clq-SP, while some investigators use Clq of another species for Clq-SP (Ziola et al. 1982; Lin et al. 1983). So we used human Clq and rabbit Clq for Clq-SP and the interaction of CIC-like IgG was observed in both. It is recommended to use heat inactivated Clq as a blank in Clq-SP and subtract it as a background.

References


