ELSEVIER

Journal of Immunological Methods 249 (2001) 199–205

JIM
Journal of
Immunological Methods

www.elsevier.nl/locate/jim

An improved anti-C3/IgG ELISA for quantification of soluble immune complexes

Neci Matos Soares[a,b,*], Mittermayer B. Santiago[c], Lain C. Pontes deCarvalho[a,c]

[a]*Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcao 121, 40295-001 Salvador, Brazil*
[b]*Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, Brazil*
[c]*Escola Bahiana de Medicina e Saúde Pública, Salvador, Brazil*

Received 12 September 2000; accepted 30 November 2000

Abstract

A semi-quantitative ELISA for complement-fixing, IgG-containing immune complexes (IC) is described. The assay is based on the insolubilization of IC by polyethyleneglycol, their capture by solid-phase anti-C3 antibodies, reaction with peroxidase-labeled anti-IgG antibodies and incubation with a chromogenic peroxidase substrate. It was markedly improved by the use of a single-step procedure which simultaneously washed and precipitated the insolubilized immune complexes. Intra-assay and inter-assay coefficients of variation were lower than 8.6 and 14.7%, respectively. As expected, higher levels of circulating immune complexes, in relation to healthy individuals, were found in patients with American visceral leishmaniasis (AVL), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), with prevalences comparable to those described in the literature. The ELISA can be quickly assembled from reagents and plasticware widely available commercially, detects immune complexes fulfilling three different criteria and is more sensitive than a previously published method based on the same principles (detection limit for complement-sensitized aggregated IgG of 2 $\mu g\ ml^{-1}$ as compared with a detection limit above 16 $\mu g\ ml^{-1}$).

Keywords: Immune complexes; Polyethylene glycol; ELISA; C3; IgG

1. Introduction

Immune complexes (IC) participate in the pathogenesis of many diseases and exert profound effects on humoral and cellular immune responses (Ramos-Niembro et al., 1982; Adyel et al., 1996; Berger et al., 1997; Quayle et al., 1997). The quantification of circulating immune complexes (CIC) in patients with inflammatory processes has been used both for diagnosis and determination of disease activity (Huber et al., 1989; Basson et al., 1991). There is no widespread agreement, however, on the relative efficacies of available CIC-detecting assays. This has been ascribed to difficulties in reproducibility or execution and/or to principle unsoundness of the test systems (Lambert, 1978).

In the present study, an ELISA for the semi-

Abbreviations: agIgG, complement-sensitized aggregated IgG; AVL, American visceral leishmaniasis; BB, 0.3 M borate buffer, pH 8.3; BB-milk-PEG, BB containing 6% skimmed milk and 2.5% polyethylene glycol; CIC, circulating immune complexes; IC, immune complexes; PEG, polyethyleneglycol; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus

*Corresponding author. Tel.: +55-71-356-8781; fax: +55-71-356-2255.

E-mail address: neci@cpqgm.fiocruz.br (N.M. Soares).

0022-1759/01/$ – see front matter
PII: S0022-1759(00)00362-8

quantification of soluble immune complexes was developed. To produce a signal in it, the complexes had to fulfill three different criteria, namely to be insoluble in 2.5% polyethylene glycol (PEG) and to contain IgG and C3 epitopes.

The ELISA resulted from a combination of two previously published assays (Pontes de Carvalho et al., 1986; Xu and Gu, 1990), preserving their best features. Radiation hazards were avoided by the use of peroxidase-labeled instead of radiolabeled antibodies; a one-step centrifugation procedure was used to wash insolubilized CIC, minimizing losses and variability due to multiple centrifugations. The assay was demonstrated herein by its application to the detection of CIC in patients with American visceral leishmaniasis (AVL), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).

2. Materials and methods

2.1. Human sera

Sera from 53 patients with AVL (with diagnoses confirmed by direct identification of the parasite), 85 with SLE and 14 with RA, and from 74 healthy volunteers, were studied. Diagnoses of SLE and RA were made in accordance with criteria proposed by the American Rheumatism Association (Arnett et al., 1988). All volunteers were previously informed on and understood the nature of the research; patient's sera consisted of small volumes (100 μl) necessarily remaining from serum aliquots obtained for clinical reasons.

2.2. Complement-sensitized heat-aggregated immunoglobulin

IgG (1 mg ml^{-1}) was purified from normal human serum by affinity chromatography on a protein A-Sepharose CL 4B column (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and aggregated by incubation at 63°C for 30 min. Complement-sensitized aggregated IgG (agIgG), used to obtain standard curves, were formed by diluting aggregated IgG in 0.15 M phosphate-buffered saline, pH 7.2 (PBS), containing 7.5% of fresh normal human serum, as a source of complement. This was stored in small aliquots at −20°C prior to use.

2.3. Insolubilization of CIC and agIgG

CIC and agIgG were insolubilized by PEG (PEG 6000; Sigma Chemical Co., St. Louis, MO), as previously described (Pontes de Carvalho et al., 1986). Briefly, 100 μl of neat serum or agIgG dilutions were mixed by inversion with an equal volume of 0.3 M borate buffer, pH 8.3 (BB), containing 0.1 M EDTA and 5% PEG, overnight, at 4°C. The mixtures were diluted 1:3 in BB containing 0.002% phenol red and 2.5% PEG, previous to being tested for the presence of insoluble CIC or agIgG, so that the final serum dilution was 1:6.

2.4. Quantification of IgG

In order to exclude the possibility that the procedure described above could insolubilize free IgG, normal human serum was treated as described with 2.5% PEG and centrifuged for different periods of time. The amounts of IgG present in the precipitates were then measured in an ELISA. The ELISA was based on the competition for an anti-IgG–peroxidase conjugate of the test IgG with microtiter well-bound IgG (Barros et al., manuscript in preparation).

2.5. One-step centrifugation procedure for both precipitating and washing insolubilized CIC and agIgG

The insolubilized complexes were simultaneously precipitated and washed by a previously described method (Pontes de Carvalho et al., 1986), slightly modified. Briefly, 900 μl of BB containing 6% powdered skimmed milk (w/v) and 2.5% PEG (BB-milk-PEG) were placed into 1.5 ml conical-bottom microcentrifuge plastic tubes (Thomas Scientific, Philadelphia, PA). Approximately 4 cm-long plastic cylinders were made out of 1000 μl automatic pipette tips (Universal Fit Pipette Tips; Corning Costar Corporation, Cambridge, MA) by cutting off their conical points. These cylinders were introduced as much as possible into the microcentrifuge tubes containing BB-milk-PEG, so that about half cylinder length remained outside the tubes, and the BB-milk-

PEG filled the cylinder lowest part up to approximately 1 cm. Subsequently, 100 μl of the red-colored serum or agIgG dilutions were placed over the BB-milk-PEG surface within the plastic cylinders, in duplicates. After centrifugation (3600×*g*, 30 min), the upper opening of the cylinders were air-tightly blocked by the assayer's index finger, who, by holding the cylinders between thumb and middle finger, withdrew them from the tubes, together with the red-colored serum or agIgG supernatants. In this way, no soluble material from the serum or agIgG dilutions ever entered the microcentrifuge tubes, precluding the need for washing the precipitates by successively filling up and centrifuging the tubes. After removing the cylinders, most of the remaining BB-milk-PEG solution was discarded by aspiration through a Pasteur pipette connected to a vacuum pump, leaving the washed precipitates and about 220 μl of BB-milk-PEG inside the tubes. The length of the Pasteur pipette that was allowed to enter the tubes was determined by a small rubber cap attached to the pipette tip (attachment was accomplished by simply leaving a perforated cap as a rubber ring around the pipette tip). Insolubilized IC from some sera were washed with BB-milk-PEG also in the classical way, i.e. by means of three centrifugations for 10 min at 15 000×*g*.

2.6. Detection of C3- and IgG-containing complexes

This was performed as follows. Wells of microtiter plates (Corning, NY) were coated with goat anti-C3 antibodies (serum globulin fraction; Sigma Chemical Co., St. Louis, MO) through overnight incubation at 4°C with 100 μl of 0.06 M sodium carbonate-bicarbonate buffer, pH 9.6, containing 30 μg ml^{-1} of the antibody preparation. After incubation, the wells were blocked by incubation for 1 h at 37°C with 100 μl of PBS containing 5% skimmed milk and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Then 100 μl of washed PEG precipitates in BB-milk-PEG were added to the wells. On each plate, a set of wells contained double dilutions of PEG-precipitated agIgG (1.25–80 μg of protein ml^{-1}) or BB-milk-PEG alone. The plates were then incubated for 1 h at 37°C and 1 h at 4°C, followed by three washings with PBS-T. Subsequently, 100 μl of a 1:5.000 dilution of peroxidase-labeled anti-human IgG (γ-chain specific; Sigma Chemical Co., St. Louis, MO) were added to each well, followed by 1 h incubation at 37°C. The plates were then washed three times as described above and 100 μl of a peroxidase substrate (ortophenylenodiamine and hydrogen peroxide) solution were added to each well. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 25 μl of 2 M H_2SO_4 and the optical density (OD) read at 490 nm. Results were expressed as equivalents to μg of agIgG ml^{-1} (eq. μg agIgG ml^{-1}). Values above the mean of the results obtained from 75 healthy individuals' sera plus three standard deviations of the mean were considered as being positive.

2.7. Comparison between two anti-C3/IgG assays for immune complexes

In accordance with a previously published protocol (Xu and Gu, 1990), increasing amounts of agIgG were insolubilized in 2000 μl of a 1:40 human serum dilution containing 2.5% of PEG. Also following Xu and Gu's methodology (Xu and Gu, 1990), the precipitates obtained after centrifugation were not washed. The same amounts of agIgG were tested in parallel in the assay described herein, which uses a one-step washing procedure and much smaller volumes (100 μl) of less diluted (1:6) serum. The precipitates were then assayed for the presence of complexes containing both C3 and IgG epitopes as described above.

3. Results

3.1. Detection of different amounts of aggregated IgG by anti-C3/IgG ELISA

AgIgG in amounts varying from 1.25 to 80 μg was diluted with PBS containing 7.5% normal human serum and 100 μl aliquots were tested in the assay in duplicates. There was a linear relationship between added agIgG and the optical densities obtained in the ELISA (Fig. 1). Wells coated with bovine serum albumin or normal goat IgG instead of anti-C3 produced optical densities similar to wells in

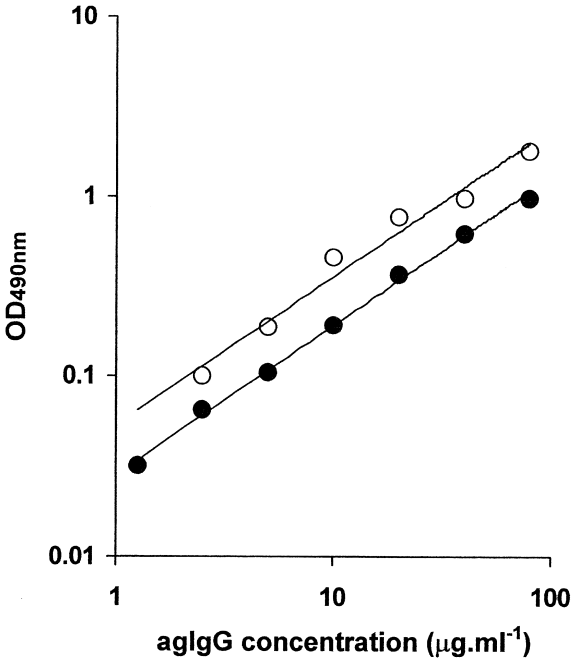

Fig. 1. Detection of increasing amounts of aggregated IgG (agIgG) by an anti-C3/IgG ELISA. Fresh normal human serum containing the indicated concentrations of agIgG was tested in the ELISA as described in the Materials and methods section. Each symbol corresponds to the mean optical density at 490 nm ($OD_{490\ nm}$) of duplicates. The two depicted regression lines correspond to independently obtained sets of results.

which no serum or agIgG was added (ranging from 0.034 to 0.039).

3.2. Assessment of IgG solubility in 2.5% of PEG

False–positive results could be produced in the assay described above if free IgG with rheumatoid factor activity was precipitated by 2.5% PEG, since it would recognize as antigen the heterologous anti-C3 antibodies on the solid phase and, in its turn, be recognized as antigen by the anti-IgG-peroxidase conjugate. However, only a very small amount of the IgG present in normal human serum precipitated in the presence of 2.5% PEG, notwithstanding increasingly centrifugation times, showing that the bulk of the IgG, i.e. normal IgG, does not precipitate with 2.5% PEG under the conditions of the assay (99.45% of the IgG remained in the supernatant after 5 min of centrifugation, 99.03% after 10 min, 98.86% after 15 min, 98.94% after 20 min and 99.06% after 30 min).

3.3. Comparison between two washing procedures for insoluble immune complexes

To find out whether the one-step washing procedure produced better results than a classical three-centrifugation washing procedure, 10 AVL patient's sera and two normal human sera were tested employing each type of washing in parallel. The one-step washing procedure led to significantly higher IC levels in the patients' sera than the three-centrifugation washing procedure ($P<0.005$; Wilcoxon's non-parametric ranking test for paired samples), without increasing the values obtained with the normal sera (Fig. 2).

3.4. Reproducibility of the assay

The inter-assay coefficient of variation, obtained by testing three sera in five different assays, was found to be 14.7% for a high (640 ± 94 μg ml^{-1}),

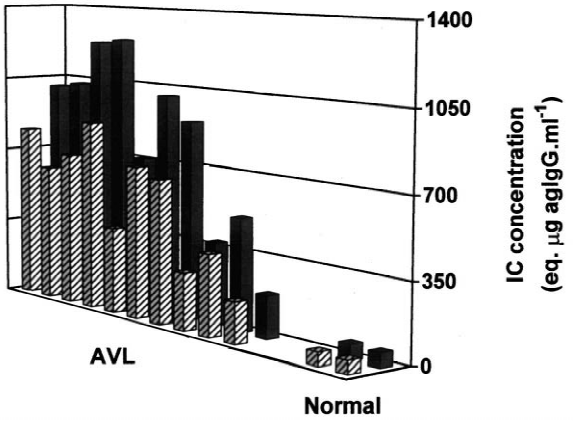

Fig. 2. Effect of two different washing procedures on the concentrations of immune complexes (IC) as determined by anti-C3/IgG ELISA. IC were precipitated from 1:6-diluted sera, washed either by a single-step (solid columns) or a classical three-step (hatched columns) centrifugation procedure and detected as described in the Materials and methods section. Sera were from 10 patients with American visceral leishmaniasis (AVL) and two healthy individuals (Normal). IC amounts were corrected for the dilution factor and expressed as equivalents to μg of aggregated IgG per ml (eq. μg agIgG ml^{-1}), as described in the Materials and methods section.

7.9% for a medium (209±16.6 μg ml^{-1}) and 8.2% for a low (58.2±4.8 μg ml^{-1}) concentration of CIC. To determine the intra-assay coefficient of variation, eight replicates of two sera were tested in the same assay. It was found to be 8.6% for a medium (225.5±19.5 μg ml^{-1}) concentration and 7.1% for a low (46.6±3.3 μg ml^{-1}) concentration of CIC.

3.5. Comparison between two anti-C3/IgG assays for immune complexes

The assay employing the single-step washing procedure for PEG precipitates was able to detect a variation from 1 to 2 μg ml^{-1} on agIgG concentrations, whereas the assay in which the agIgG was not washed detected only variations larger than 16 μg ml^{-1} (Fig. 3).

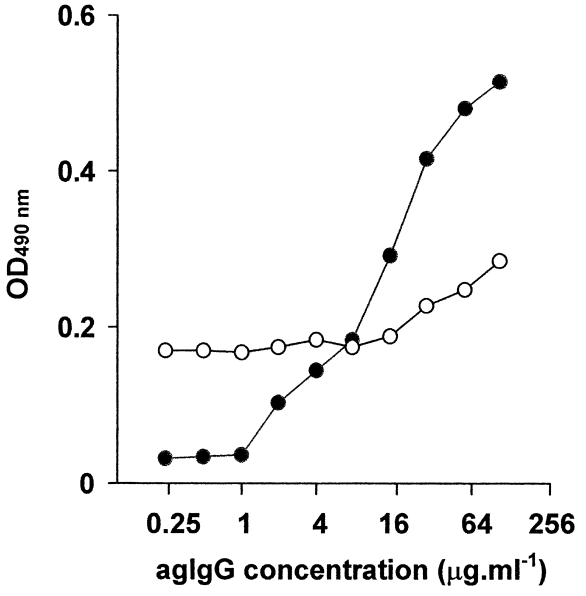

Fig. 3. Comparison between two anti-C3/IgG ELISA for soluble immune complexes. Fresh normal human serum containing the indicated concentrations of aggregated IgG was tested in the ELISA described in this paper (solid symbols), which uses a single-step washing procedure for polyethylene glycol precipitates, and in an ELISA using unwashed precipitates (open symbols), in accordance with a previously published method (Xu and Gu, 1990), as described in the Materials and methods section. Each symbol corresponds to the mean optical density at 490 nm ($OD_{490\ nm}$) of duplicates.

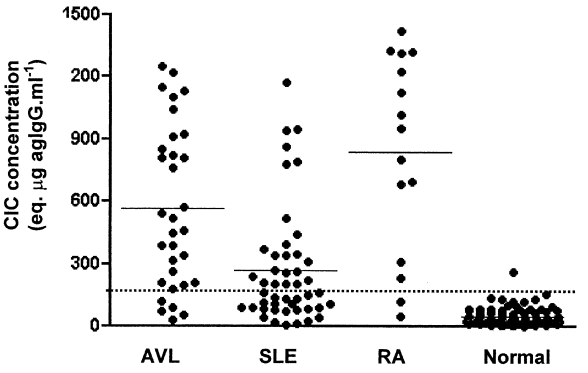

Fig. 4. Circulating immune complex (CIC) levels in 34 patients with American visceral leishmaniasis (AVL), 50 patients with systemic lupus erythematosus (SLE), 14 patients with rheumatoid arthritis (RA) and 75 healthy individuals (Normal). Each symbol represents the mean value of duplicates. The horizontal full lines represent group means, and the broken line represents the mean value of the results obtained from 75 normal human sera plus three standard deviations of the mean. IC amounts were measured in 100 μl volumes of 1:6-diluted serum, corrected for the dilution factor and expressed as equivalents to μg of aggregated IgG per ml (eq. μg agIgG ml^{-1}), as described in the Materials and methods section.

3.6. Detection of CIC in patients with different diseases

The assay disclosed significantly higher levels (Fig. 4) and prevalence (Table 1) of CIC in patients with AVL, RA or SLE in relation to a normal control group.

4. Discussion

Most assays employed for quantifying CIC have important drawbacks, leading to widely discrepant percentages of patients in whom CIC are detected (Lambert, 1978). This has been ascribed to variations in CIC sizes and in their contents of antibodies, antigen and/or complement (Baldwin et al., 1982; Larsson and Sjoquist, 1988; Nezlin and Mozes, 1995). Moreover, immune complexes continuously change their size and nature in vivo, since they may release non-covalently bound antigen or complement factors. The complement fragment C3b is not released, however, since it binds covalently to antibodies through a reactive carboxyl group.

Table 1
Quantification of circulating immune complexes (CIC) by anti-C3/IgG ELISA

Serum donor	Number of cases	CIC concentration[a]	Percentage positive[b]
Systemic lupus erythematosus patients	50	238.5±241.9[c]	44.0
Visceral leishmaniasis patients	34	581.0±379.0	88.2
Rheumatoid arthritis patients	14	803.8±474.0	76.5
Normal controls	75	51.4±44.2	1.3

[a] CIC concentrations were measured in 100 μl volumes of 1:6-diluted serum, corrected for the dilution factor and expressed as equivalents to μg of aggregated IgG per ml, as described in the Material and methods section.
[b] Sera with concentrations of CIC higher than the mean of the concentrations from 75 normal controls plus three standard deviations of the mean were considered positive.
[c] Mean value of CIC concentrations in the group±standard deviation of the mean.

In this work anti-C3 antibodies were used to detect complement-fixing CIC, which are probably the most pathogenic IC because of the pro-inflammatory properties of complement (Bourke et al., 1982). As little as 2 μg of heat-aggregated IgG per ml could be detected. This indicates a considerably higher sensitivity than that of a previously published anti-C3 ELISA, based on identical principles and using the same reagents (Xu and Gu, 1990), which could not detect any doubling in aggregated IgG concentrations up to 16 μg ml^{-1}. The relatively low sensitivity of this last assay could be due to the unavoidable contamination of unwashed precipitates by soluble C3, which would compete with the C3b of the complexes for the solid-phase anti-C3. An additional advantage of the assay described herein over the one proposed by Xu and Gu (1990) is that it does not require the preparation of $F(ab')_2$. The use of $F(ab')_2$ in the solid phase is justified in terms of avoiding false positive results due to free anti-Fc IgM rheumatoid factors, which reacts with heterologous IgG and would form bridges between the anti-C3 antibody and the anti-immunoglobulin–peroxidase conjugate. These factors do not interfere with the present assay for two reasons: (1) a δ chain-specific anti-IgG conjugate is used; and (2) free IgM rheumatoid factor does not precipitate in 2.5% PEG (Baldwin et al., 1982). PEG-precipitable IC containing IgM rheumatoid factor, its IgG antigen and C3b would of course be appropriately detected in both assays.

The one-step washing procedure also produced better results than a classical three-step washing procedure, increasing the amounts of IC detected in AVL patients' sera and not affecting the results obtained with normal human sera. This could be explained by a probable loss of immune complexes due to multiple washings.

The percentages of patients with AVL (88%), SLE (44%) and RA (77%) that had CIC as determined by the anti-C3/IgG ELISA reported herein is consistent with data reported in the literature. For instance, percentages of 89% for Old World visceral leishmaniasis (Azazy et al., 1994), 41% for SLE (Bukh et al., 1988) and 54% for RA (Bourke et al., 1982; Huber et al., 1989) have been reported.

The concentration of PEG used in this assay (2.5%) does not precipitate IgG-C3 complexes (Xu and Gu, 1990) and free IgG (this paper). The 2.5% PEG-precipitated material containing IgG and C3 epitopes detected in the assay described herein, therefore, can only be ascribed to CIC.

The ELISA described herein has one or more of the following advantages over other available assays: (1) CIC have to fulfill three different criteria in order to be detected; (2) it uses a virtually failure-proof procedure for washing insolubilized IC, which avoids both contamination with soluble material due to insufficient washing and loss of IC due to aspiration of supernatants or adsorption to centrifuge tube walls; (3) it detects only complement-fixing, potentially pathogenic immune complexes; and (4) it uses relatively inexpensive and widely available reagents and plasticware.

References

Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S., 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 31, 315.

Adyel, F.Z., Hentati, B., Boulila, A., Hachicha, J., Ternynck, T., Avrameas, S., Ayadi, H., 1996. Characterization of Autoantibody activities in sera anti-DNA antibody and circulating immune complexes from 12 systemic lupus erythematosus patients. J. Clin. Lab. Anal. 10, 451.

Azazy, A.A., Devaney, E., Chance, M.L., 1994. A PEG-ELISA for detection of *Leishmania donovani* antigen in circulating immune complexes. Trans. R. Soc. Trop. Med. Hyg. 88, 62.

Baldwin, W.M., Van Es, A., Valentijn, R.M., Van Gemert, G.W., Daha, M.R., Van Es, L.A., 1982. Increased IgM and IgM immune complex-like material in the circulation of renal transplant recipients with primary cytomegalovirus infections. Clin. Exp. Immunol. 50, 515.

Basson, J., Tardy, J.C., Aymard, M., 1991. Characterization of immune complexes containing cytomegalovirus-specific IgM antibodies following a kidney graft. J. Med. Virol. 33, 205.

Berger, S., Chandra, R., Balló, H., Hildenbrand, R., Stutte, H.J., 1997. Immune complexes are potent inhibitors of interleukin-12 secretion by human monocytes. Eur. J. Immunol. 27, 2994.

Bourke, B.E., Moss, I.K., Mumford, P., Horsfall, A., Maini, R.N., 1982. The complement fixing ability of putative circulating immune complexes in rheumatoid arthritis and its relationship to extra-articular disease. Clin. Exp. Immunol. 48, 726.

Bukh, A., Thomsen, B.S., Kimose, H.H., Nielsen, L.R., Svehag, S.E., Moller, N.P., 1988. A polyclonal IgM-RF enzyme-linked immunosorbent assay for the detection of circulating immune complexes. J. Clin. Lab. Immunol. 26, 195.

Huber, C., Ruger, A., Herrmann, M., Krapf, F., Kalden, J.R., 1989. C3-containing serum immune complexes in patients with systemic lupus erythematosus: correlation to disease activity and comparison with other rheumatic diseases. Rheumatol. Int. 9, 59.

Lambert, P.H., 1978. A WHO collaborative study for the evolution of eighteen methods for detecting immune complexes in serum. J. Clin. Lab. Immunol. 1, 1.

Larsson, A., Sjöquist, J., 1988. Quantification of circulating immune complexes by chicken anti-C4 micro ELISA. J. Clin. Lab. Immunol. 27, 39.

Nezlin, R., Mozes, E., 1995. Detection of antigens in immune complexes by a dot blot assay. J. Immunol. Methods 184, 273.

Pontes de Carvalho, L.C., Lannes-Vieira, J., Giovanni-de-Simone, S., Galvão-Castro, B., 1986. A protein A-binding, polyethylene glycol precipitation-based immunoradiometric assay. J. Immunol. Methods 89, 27.

Quayle, J.A., Watson, F., Bucknall, R.C., Edwards, S.W., 1997. Neutrophils from the synovial fluid of patients with rheumatoid arthritis express the high affinity immunoglobulin G receptor, FcγRI (CD64): role of immune complexes and cytokines in induction of receptor expression. Immunology 91, 266.

Ramos-Niembro, F., Fournié, G., Lambert, P.H., 1982. Induction of circulating immune complexes and their renal localization after acute or chronic polyclonal B-cell activation in mice. Kidney Int. 21, 29.

Xu, R., Gu, Y., 1990. Detection of circulating immune complexes with polyethylene glycol precipitation $F(ab')_2$ anti-C_3 ELISA. J. Immunol. Methods 135, 225.