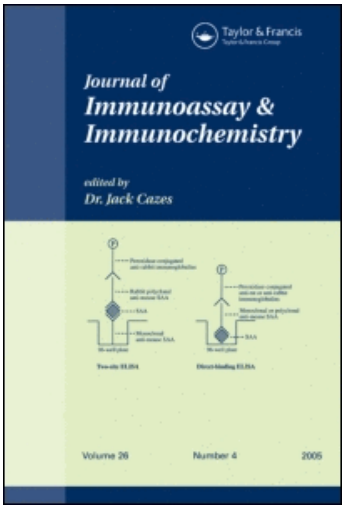

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 31 July 2001

To cite this Article Sivasankar, B. , Raju, K. R. , Ayub, S. , Srivastava, L. M. and Das, N.(2001) 'VALIDATION OF AN ELISA FOR THE QUANTITATION OF HUMAN COMPLEMENT RECEPTOR 1', Journal of Immunoassay and Immunochemistry, 22: 3, 289 — 297
To link to this Article: DOI: 10.1081/IAS-100104712
URL: http://dx.doi.org/10.1081/IAS-100104712

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 22(3), 289–297 (2001)

VALIDATION OF AN ELISA FOR THE QUANTITATION OF HUMAN COMPLEMENT RECEPTOR 1

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ABSTRACT

An ELISA was developed and validated for the quantitation of Complement Receptor 1 (CR1) in human plasma. The ELISA employed a monoclonal anti-CR1 antibody adsorbed onto microtiter plates to capture CR1 in human plasma. The captured CR1 was treated with a detecting antibody which had a different epitopic specificity for CR1. HRP conjugated anti IgG (secondary antibody) was used for quantitation.

The standard curve covered a wide range from 10 pg to 800 pg. The inter- and intra-assay variation were found to be low and within the acceptable limits. Specificity and accuracy for the assay was established by ensuring negligible cross reactivity with other proteins and an excellent parallelism between the sample and standard curve. The samples were checked for loss of sCR1 levels through freeze/thaw cycles at different intervals of time stored at -70°C.

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INTRODUCTION

Complement Receptor 1 (CR1/CD35) is a intrinsic membrane glycoprotein found on a variety of cells such as erythrocytes, polymorphonuclear leukocytes, monocytes, B lymphocytes, T lymphocytes, mast cells, and renal podocytes.(1) This protein is endowed with a dual function of inactivating the complement cascade as a complement regulatory protein and clearance of immune complexes from circulation. We have earlier reported an assay system to quantitate the surface expression of CR1 on erythrocytes.(2) CR1 is also present in the plasma in a soluble form (soluble CR1 or sCR1), which is indistinguishable in size and antigenicity from erythrocyte CR1.(3) Though the function of CR1 is not clear, the levels of sCR1 in various disease conditions is known to go high.(4,5) Specific assay kits to quantify sCR1 in human plasma samples are not commercially available.

The present study was undertaken to develop and validate a simpler and sensitive sandwich ELISA for sCR1 in order to evaluate the diagnostic and prognostic significance of sCR1 in-patients with acute renal failure.

EXPERIMENTAL

Materials

Monoclonal antibodies to human CR1 developed in mouse was a gift from M. D. Kazatchkine (France). Monoclonal antibodies to human CR1 developed in rabbit was a gift from M. R. Daha (The Netherlands). HRP conjugated anti-rabbit IgG was obtained from Dako. Recombinant CR1 was a gift from J. P. Atkinson (USA). High binding ELISA plates were from Costar.

Checkerboard Assay

The optimum dilution of the detecting antibody and the conjugate to be used in the ELISA was determined by the checkerboard assay.(6) High binding ELISA plates were coated with rabbit monoclonal antibody to human CR1 (100 µl) at dilutions of 1:250, 1:500, 1:1000, 1:2000, and 1:4000 in 50 mM carbonate bicarbonate buffer, pH 9.6, and incubated at 37°C for 2 hours. At the end of the incubation, the wells were washed thrice with 0.01 M PBS-Tween (0.5%). Unbound sites were saturated with

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2% BSA-PBS-Tween (250 μL) for 1 hour at 37°C. Plates, after thorough washing, were incubated with HRP conjugated anti-rabbit IgG (100 μL) at dilutions of 1:250, 1:500, 1:1000, 1:2000, and 1:4000 at 37°C for 1 hour and washed with PBS-Tween. Finally, 100 μL of substrate (OPD 4 mg/well in 0.1 M citrate phosphate buffer pH 5.0) was added to each well. The enzymatic reaction was allowed to proceed for 30 minutes and then stopped with 50 μL of 0.2 N H_2SO_4. The optical density (OD) was measured using an ELISA reader (Anthos) at 492 nm.

Standard Curve

ELISA was developed by a modification of the method described by Schifferli et al.(4) Microtiter plates were coated with mouse monoclonal antibody to human CR1 (0.3 μg/well) in 50 mM carbonate bicarbonate buffer, pH 9.4, and left overnight at 4°C. Plates were emptied by inverting. Unbound sites were saturated with 2% BSA-PBS-Tween for 2 hours at 37°C. After washing the plates, 100 μL of recombinant CR1, at concentrations ranging from 10 pg to 1000 pg, were added to the respective wells and left overnight at 4°C. After washing, monoclonal rabbit antibody to human CR1, diluted 1:500 times, were added (100 μL). After incubating for 3 hours at 37°C, the plates were washed and incubated with 100 μL of HRP conjugated anti-rabbit IgG (1:500) for 1 hour at 37°C. Plates were washed and treated with 100 μL of substrate (4 mg/mL OPD in 0.1 M citrate phosphate buffer, pH 5.0) in each well. The enzymatic reaction was allowed to proceed for 30 minutes and then stopped with 50 μL of 0.2 N H_2SO_4. The absorbance was measured in an ELISA reader (Anthos) at 492 nm. A standard curve was drawn.

Specificity of the Assay

Specificity of the assay was checked by

(i) performing the cross reaction studies with gelatin and human serum albumin,
(ii) checking non-specific binding between HRP conjugated anti-rabbit IgG and mouse monoclonal antibody to human CR1 (Capture antibody), and
(iii) checking non-specific binding between HRP-conjugated anti-rabbit IgG and rabbit monoclonal antibody to human CR1 with BSA.

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Collection and Storage of Human Samples

Heparinized peripheral venous blood samples from normal healthy controls of different age groups were collected. Plasma was separated by centrifugation and stored at −70°C for various time periods up to 6 months and assayed. Stability of the samples was checked at different intervals of time for loss of sCR1 levels through freeze/thaw cycles.

Parallelism with the Standard Curve

One of the samples, containing high concentration of sCR1, was sequentially double-diluted and assayed for sCR1 by ELISA. This was done to demonstrate that samples with sCR1 concentrations greater than the upper limit of the ELISA standard curve can be accurately predicated when diluted into any region of the curve.

Intra- and Inter-assay Coefficient of Variation

To evaluate the precision of the assay, three samples containing low, medium, and high concentrations of sCR1 were assayed in five replicates in six different experiments. Inter-assay and intra-assay variations were then calculated.

Quantitation of Normal Human Samples by ELISA

The levels of sCR1 in the plasma samples were quantitated by ELISA using the protocol described earlier for the standard curve except that, instead of standards, 100 μL of samples of various dilutions were added to the sample wells. The value for CR1 in each sample was calculated from the standard curve which was run simultaneously.

RESULTS

Checkerboard Assay

Based on the optical density values, an optimal antibody dilution of 1:500 was selected for both rabbit monoclonal antibody to human CR1 and HRP conjugated anti-rabbit IgG (data not shown). This dilution was used further for developing the ELISA.

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Standard Curve

The composite standard curve (Fig. 1) of six different ELISAs, conducted at different time periods, show a sensitivity of 10 pg. The maximum range of the assay was 800 pg.

Cross-reactivity of the Antisera

Cross-reaction studies revealed the ELISA to be very specific, having little cross-reactivity with gelatin and human serum albumin (Fig. 2). Rabbit monoclonal antibody to human CR1 and HRP conjugated anti-rabbit IgG showed no non-specific binding to BSA. HRP conjugated anti-rabbit IgG did not show any non-specific binding to the capture antibody (mouse monoclonal antibody to CR1).

Stability

No significant variation in the stability of sCR1 was observed upon storage at $-70^{\circ}C$ till six months, with intermittent freezing and thawing.

Sample Dilution Curve and Parallelism with Standard Curve

Double dilution of the sample showed excellent parallelism with the standard curve and the optimal concentration of samples to be used was found to be 5 to 40% (Fig. 3).

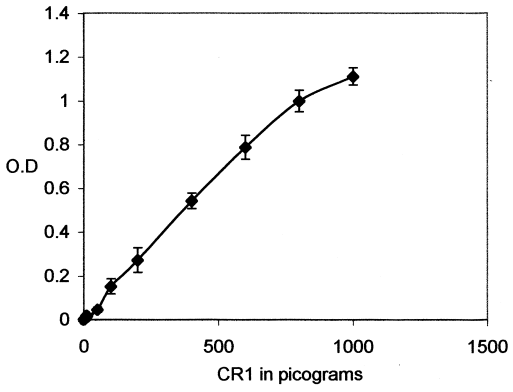

Figure 1. Composite standard curve of six different assays carried out at different times.

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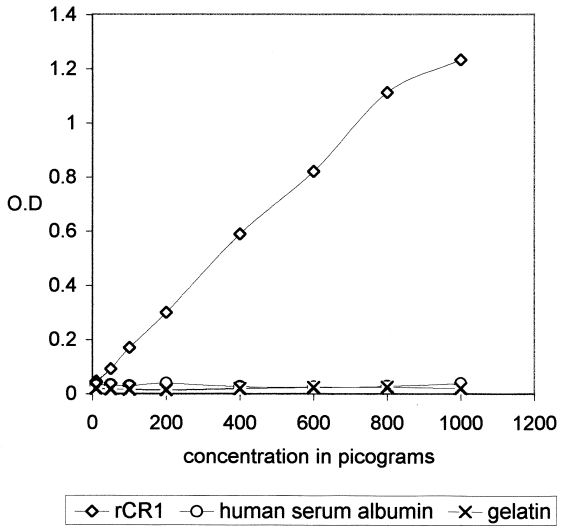

Figure 2. Cross-reactivity of CR1 antibodies to other proteins.

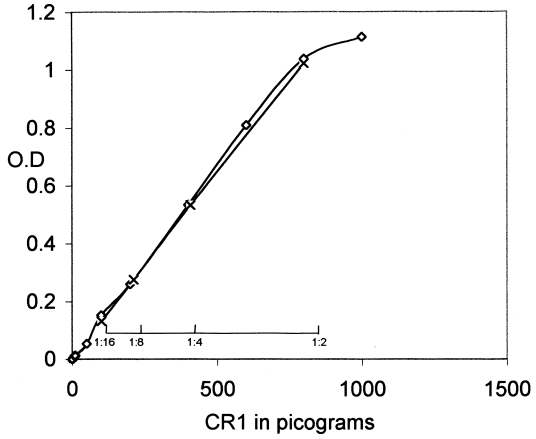

Figure 3. Parallelism between the sample and the standard dilution curve.

Intra- and Inter-assay Variation

The intra- and inter-assay variation of six assays have been evaluated to assess the precision of the method. Table 1 shows the mean, standard deviation, and coefficient of variation of each of three sample pools.

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Table 1. Intra and Interassay Variations of the Sample Values Determined by the ELISA

Expt. No.	No. of Replicates	Low		Medium		High	
		Mean ± SD	CV %	Mean ± SD	CV %	Mean ± SD	CV %
Intra-assay Variation							
1	5	23.80 ± 0.95	4.01	85.96 ± 2.07	2.40	143.86 ± 1.00	0 .69
2	5	22.46 ± 1.18	5.28	83.34 ± 2.45	2.93	147.92 ± 1.99	1.34
3	5	22.68 ± 1.68	7.44	85.70 ± 2.29	2.67	143.54 ± 3.20	2.22
4	5	24.94 ± 2.38	9.57	85.48 ± 2.83	3.31	144.30 ± 3.77	2.61
5	5	24.82 ± 1.69	6.83	86.90 ± 2.80	3.22	146.72 ± 3.14	2.14
6	5	23.06 ± 0.93	4.06	85.20 ± 1.67	1.96	145.36 ± 1.49	1.02
Inter-assay Variation							
		23.62 ± 1.475	6.19	85.43 ± 2.35	2.74	145.28 ± 2.43	1.67

The intra-assay coefficient of variation was maximum (6.19) at low concentration while, at high concentration, it was less (1.67).

Normal Range in Healthy Individuals

The sCR1 levels quantitated from plasma of healthy individuals ranged from 25 to 74 ng/mL with a mean value of 44.69 ± 12.50.

DISCUSSION

The objective of these studies was to develop and validate a sandwich ELISA for sCR1 to support clinical studies. Towards this end, a sensitive, precise, accurate, and robust assay was developed for the quantitation of sCR1 in plasma. The ELISA method utilized a mouse monoclonal anti-CR1 capture antibody with a rabbit monoclonal anti-CR1 antibody for detection. An HRP conjugated anti-rabbit IgG was used as a secondary antibody to develop color with OPD as substrate.

The optimal dilution of the detection antibody and its secondary antibody was decided based on the checkerboard assay. The standard curve showed a range of reliable response from 10 to 800 pg with minimum detectable limit of 10 pg . Low cross-reactivity with human serum albumin and gelatin ensures the specificity of this ELISA. Further low cross reactivity between the capture antibody and the secondary antibody rules

out the possibility of false positive values. Detecting antibody and secondary antibody showed little reactivity with BSA, the blocking agent. Quantitation of sCR1 using this ELISA indicated that sCR1 remained stable even after six months of storage at −70°C. Freeze/thaw cycles did not affect the stability. These stability data are needed to provide guidelines to the various clinical studies to ensure proper collection, storage, and shipment of clinical samples to the analytical site.

The experiments to evaluate the linearity of dilution were carried out to ensure that clinical samples which contain sCR1 concentrations exceeding that of the highest concentration for the standard, could be accurately quantified following appropriate dilutions. 5 to 40% of the sample showed excellent parallelism with the standard curve. Evaluation of the quality control data in all validation runs carried out showed that the ELISA is robust, precise, accurate, and reproducible, with standard deviation and coefficient of variation 1.475, 6.19% (low), 2.35, 2.74% (medium), and 2.43, 1.67% (high), respectively.

Using this ELISA, sCR1 levels in normal controls was found to range from 25 to 75 ng/mL. In conclusion, a sandwich ELISA assay for quantitation of sCR1 in human plasma was developed and validated to support the clinical studies for sCR1.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support by the Council for Scientific and Industrial Research and the Department of Science and Technology. Address for reprints: Dr. Nibhriti Das, Additional Professor, Department of Biochemistry, All India Institute of Medical Sciences, New Delhi 110 029, India.

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Received October 2, 2000 Manuscript 3017
Accepted November 12, 2000

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