CR1 BINDING ASSAY: A NOVEL ELISA ASSAY FOR MEASURING CIRCULATING IMMUNE COMPLEXES

Erick K Mibei, PhD
School of Health Sciences, University of Kabianga, Kericho, Kenya
Salinah J Rono, MSc
Department of Biological Sciences, University of Kabianga, Kericho, Kenya
Jose Stoute, MD,
Dept. of Medicine, Penn State University College of Medicine Hershey,USA
John N Waitumbi, PhD
The US Army Medical Research Unit, Kisumu, Kenya

Abstract

There are over fifty methods which have been described for the assessment of immune complexes in sera or plasma. The measurement of circulating immune complexes in sera of patients with different diseases has been frequently used for the assessment of disease activity for the purposes of disease management/therapy and control. Complement 1q (C1q) binding assay has been the most widely used. In the present study circulating immune complexes (CIC), were measured for the purposes of assessing the relationship with the severity of *Plasmodium falciparum* severe malarial anaemia. Three methods were used, with two being C1q-based assays and a novel CR1 binding assay. The ability of the methods to discriminate between the severe malarial anaemia cases and their controls was assessed with reference to a common heat aggregated human IgG standard. The three methods used showed a similar trend in detection of levels of CIC in sera and all methods showed elevated levels of CIC in sera of severe malarial anaemia cases compared to their controls. When the cases were compared to their symptomatic controls, the commercial Quidel C1q kit showed a significance difference between SA and UM of $p = 0.04$ while C1q in-house assay showed a highly significant difference for the two groups ($p = 0.01$). CR1 binding assay also showed a significance difference between SA and UM ($p = 0.04$). When cases were compared to their symptomatic controls, Quidel C1q kit had $p = 0.001$, C1Q in-house assay had $p = 0.006$ while CR1 binding assay had $p = 0.01$.

Their level of detection differed and this can be attributed to different tracing principles and the nature or properties of the immune complexes detected. From the results it shows that for the screening of CIC in sera, the use of two or more methods will enable detection of ICs of various sizes and complexities, and the nature of IC should be considered so as to select the most appropriate method that will enable a valid assessment to be carried out

Introduction

The assessment of immune complexes (ICs) in sera or plasma of patients has been employed for purposes of intervention and management of diseases associated with generation of high levels of ICs (Krauledat *et al*., 1985), which may persist in circulation and thus modify the cause of infection. Over fifty methods have been described for the measurement of CICs in humans and animals (Jones *et al*., 1982). Variability in sensitivity, complexity, clinical applicability and limited specificity of the available test systems complicates the choice of assay used. Thus, it is well known that the application of different assay systems for the detection of CIC will lead to divergent results due to different biological and/or physical principles. The test systems to be used should be based on different detection principles, which will permit the detection of ICs of various sizes and complexity (Krauledat *et al*., 1985).

Most test systems used are based on the complement system and its breakdown products. This is mainly because most ICs share a common property in their ability to activate the complement system via classical or alternative pathways hence it seems relevant to test its breakdown products. Most of these test systems are antigen non-specific and identify the presence of ICs by relying on the different physiochemical and biological properties of antigen-complexed antibody as opposed to free antibody (Jones *et al*., 1982). C1q binding of ICs is an indication for the classical method of complement activation. C1q binds weakly to monomeric IgG1, IgG2, IgG3 and IgM. Binding is enhanced when the proteins aggregate because of heating or antigen binding. The C1qBA detect only those ICs that bind the first component of complement. However, C1qBA detects neither ICs with non-complement activating antibodies, like IgG4, nor ICs with antibodies that activate complement preferentially via the alternative pathway (IgE and IgA) (Theofilopoulus, 1980). C1q also interacts preferentially with ICs larger than 19S. It has also been shown that C1qBA is less sensitive in measuring smaller (antigen excess) than large complexes (antibody excess) and is efficient at detecting complexes of intermediate size (between 19S and 7S)

(Woodroffe *et al*., 1977). Solid phase C1qBA is very sensitive (1-10μg/ml aggregated human IgG) and has the distinct advantage over fluid phase C1q tests in that it is antibody specific and has minimum false positive results (Mitchell *et al*., 1987).

Patients with severe malarial anaemia have been shown to have low levels of CR1 (Waitumbi *et al.,* 2000). This suggests that complement is being activated hence CR1 is being consumed. Since CR1 is required for IC clearance, low levels of CR1 lead to high level of CIC. Studies in malaria have also suggested that CIC cause hemolysis and/or erythrophagocytosis by immune adherence phenomenon through C3b receptors (Mohamed, 1982, Waitumbi *et al*., 2000).

The above observations point to the possibility that during *P. falciparum* malaria infection, ICs with the ability to fix complement are generated and this could account for hemolysis. So a method, which would enable quantification of this portion of ICs, was appropriate.

Complement receptor type 1 (CR1, CD35, C3b/C4b receptor) is a transmembrane glycoprotein expressed on several circulating cells including erythrocytes, neutrophils, monocytes/macrophages, B-lymphocytes and some T-lymphocytes (Ahearn and Fearon, 1989). CR1 on erythrocytes binds C3b and C4b and this allows erythrocytes to bind complement opsonized immune complexes (IC), a phenomenon referred to as immune adherence. This phenomenon aids in the transport of CIC from the circulation to the reticuloendothelial system including the liver and skin (Miyaike *et al*., 2002, Cosio *et al.,* 1990). This prevents the indiscriminate IC driven complement activation that might be associated with tissue injury (Cornacoff *et al*., 1983, Birmingham 1995). This ability of CR1 to bind C3b, which is an opsonin and hence is expected to coat ICs, which fix complement, was utilized in this study by immobilizing soluble CR1 onto surfaces of Elisa plate wells and will capture opsonized ICs out of a test serum.

Materials and Methods
Study design and patient population

This study was reviewed and approved by Kenya National Ethical Review Committee. The recruitment of children and study procedures were in accordance with the regulations of the Kenya Medical Research Institute. Informed consent was obtained from all parents or legal guardians.

The study reported here was case-control in design and was interested in complicated malaria pathogenesis and whose findings were reported earlier (Stoute *et al.,* Mibei *et al.,* 2005).The immune complex measurements relied on C1q binding assay for the reported studies. The demographics of the cases of severe anaemia (SA) and their controls were reported earlier (Stoute *et al*., 2003, Mibei *et al.,* 2005). SAs were defined as children with

asexual *P. falciparum* parasitemia by Giemsa-stained blood smear and hemoglobin ≤ 5 g/dL, were recruited from the Pediatric Ward of the Nyanza Provincial General Hospital (NPGH), Kisumu. The NPGH catchment area is a malaria holoendemic region of the Lake Victoria basin, western Kenya. Because this study also included a red cell component that was reported elsewhere (Stoute *el al*., 2003), children were excluded if they had a history of blood transfusion within three months preceding enrollment. In addition, cases were excluded if there was clinical evidence of other concomitant infections or malignancy. Controls with uncomplicated clinical malaria, matched by age ± 2 months and gender to each SA case, were recruited from the outpatient clinic of the hospital where the respective case was enrolled. Inclusion criteria for controls were a Giemsa-stained blood smear positive for asexual *P. falciparum* and an axillary temperature ≥ 37.5 ^{o}C or, in the absence of the latter, two of the following signs or symptoms: nausea/vomiting, irritability, poor feeding, myalgias, or headache. The majority (>80%) of the children in the control groups qualified on the basis of an axillary temperature >37.5 ^{o}C. Exclusion criteria for controls were the same as for cases with the addition of any evidence of malaria complication manifested by respiratory distress, palmar or conjuctival pallor, hypotension, seizures, hemoglobin ≤5 g/dL, or coma. As part of other ongoing studies SAs and their controls were followed at monthly intervals for a period of 3 to 6 months.

Collection and processing of blood samples

For cases, and controls thick and thin blood films were prepared from a pinprick blood and stained with Giemsa stain. Diagnosis of *P. falciparum* parasitemia was confirmed microscopically and venous blood was collected into microtainers and allowed to clot for 1 hour at room temperature. The serum was collected, aliquoted into cryovials and transported to the laboratory on dry ice packs and was frozen at –70 °C until use.

Preparation of heat aggregated IgG (AHG) standards

AHG was prepared from purified human IgG (Sigma Chemical Co., USA). 6.5mg of human IgG was reconstituted in 1ml of phosphate buffered saline (Sigma Chemical Co., USA) and this was heated at 63°C for 30 minutes. AHG was then separated by column chromatography through sephacryl S-300 2.6x70cm column (Armesham Pharmacia, Biotech, Piscataway, NJ). Fractionation was performed at a flow rate of 3ml/minute with 3.5ml fractions collected. 100µl of each fraction was pipetted onto wells of a UV microtitre plate. Absorbance was measured at 280nm using a Bioassay reader (Perkin Elmer Norwalk CT USA). Optical density values were plotted against the fractions and fractions making up peak A (Figure 2)

were pooled and protein concentration was measured by protein assay (Pierce, Rockford, ILL). 10µl of AHG sample was added to 200µl of the Pierce reagent. This was then incubated at 37°C for 30 minutes and optical density was read at 562nm using a Bioassay reader (Perkin Elmer Norwalk CT USA). Protein concentration was adjusted to 300µg/ml.

Measurement of Circulating Immune Complexes
C1q Binding Assay

To measure CICs, a commercially available kit (Quidel, Berchworth, UK) and an in-house-developed assay were used. The commercial kit comes pre-coated and ready to use. For the in-house assay, wells of an Immulon HB 96-well plate (Thermo Labsystems Helsinki, Finland) were coated with 10 µg/ml C1q (Sigma-Aldrich, St. Louis, MO) in PBS pH 7.4. After overnight incubation at 4 °C, the plates were washed with wash buffer (PBS/0.5% Tween) and blocked for 1 hour at room temperature with blocking buffer (PBS, 0.5% boiled casein, 1% Tween, 0.01% Thimerosal, 20 µg/ml phenol red). Aggregated IgG, prepared as described above, was serially diluted in PBS to get dilutions from 0-30µg/ml. Each AHG dilution and test sera were then diluted to a final dilution of 1:60 in dilution buffer (PBS, 0.5% boiled casein, 0.5% Tween, 0.01% Thimerosal, 20 µg/ml phenol red). 100 µl of diluted standard and unknown samples were added to duplicate wells and incubated for 1 hr at room temperature. The wells were emptied and washed with wash buffer x4. HRP-conjugated goat anti-human IgG (Kirkegaard and Perry Labs, Baltimore, MD) was diluted 1:5,000 in wash buffer containing 0.5% boiled casein and 100µl was added to each well followed by 1 hour incubation at room temperature. After washing x4, 200 µl of ABTS substrate (Kirkegaard and Perry) was added to each well for 45 min followed by measurement of OD_{415nm}. IC level was expressed as µg/ml of aggregated human IgG equivalent (AHG Eq.).

CR1 Binding Assay

This is a novel assay, Elisa-based developed in-house to measure CIC. The assay is based on the property of complement receptor 1 (CR1) to bind opsonized immune complexes (Pascual and Shifferli, 1995, Medof *et al*., 1982, Fearon and Ahearn, 1989). Wells of an Immulon HB 96-well plate (Thermo labsystems Helsinki, Finland) were coated with 2.5µg/ml CR1 (AVANT Immunotherapeutics, MA, USA), in PBS pH 7.4. After overnight incubation at 4 °C, the plates were washed with wash buffer (PBS/ 0.5% Tween) and blocked for 1 hour at room temperature with 300µl blocking buffer (PBS, 0.5% boiled casein, 1% Tween, 0.01% Thimerosal, 20 µg/ml phenol red). Aggregated human IgG (AHG) was prepared as previously

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described for use as standard and was serially diluted in PBS to get dilutions from 0-12µg/ml. 10µl of each AHG dilution were then diluted with an equal volume of 10% normal human serum and incubated at 37°C for 30 minutes and further diluted to3.33% in dilution buffer (PBS, 0.5% boiled casein, 0.5% Tween, 0.01% Thimerosal, 20 µg/ml phenol red). 10µl of test serum was mixed with an equal volume of PBS and then diluted further as for the standards above.100 µl of diluted standard and unknown samples were added to duplicate wells and incubated for 1 hour at room temperature. The wells were emptied and washed with wash buffer x4. HRP-conjugated goat anti-human IgG (Kirkegaard and Perry Labs, Baltimore, MD) was diluted 1:1,000 in wash buffer containing 0.5% boiled casein and 100µl was added to each well followed by 1 hr incubation at room temperature. After washing x4, 200 µl of ABTS substrate (Kirkegaard and Perry) was added to each well for 30 min followed by measurement of OD_{415nm}. IC level was expressed as µg/ml of aggregated human IgG equivalent (AHG Eq.).

Test to confirm CR1-C3b binding

To test for CR1-C3b binding, two methods of inhibiting complement activation were employed. The standard normal human serum was heat-inactivated at 56°C for 30 minutes before mixing with AHG standards and incubated as described for normal serum above. The second method employed the use of EDTA as a complement inhibitor. AHG standards were prepared as described above and then 20µl of 0.2M EDTA was added and the standards incubated for 30 minutes. The assay then proceeded as for normal standards as described above.

Results
Size distribution of heat aggregated human IgG

Sephacryl S-300 gel separated heated human gamma globulins into two prominent peaks with distinct molecular sizes (Fig.1). The first peak (Peak A) is the fraction containing aggregated gammaglobulins (AHG) and the size is around 19S while peak B mostly contains the non-aggregated gammaglobulins with a size of about 7S.

The fractions making up peak A were pooled and diluted accordingly and this is the one used as the standard for the assays.
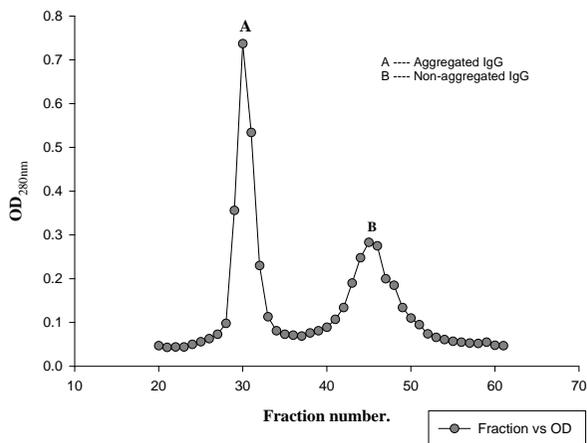
Figure 1: Elution profile of heat aggregated IgG from Sephacryl S-300. The black dots represent the fractions; peak A comprises the fractions, which contains aggregated IgG while Peak B contains the non-aggregated IgG fractions.

By using serial dilution of AHG, standard curves for Quidel C1q, in-house C1q and CR1 binding assays were generated and were used to calculate the IC equivalent concentration in the test samples (Figure 2).
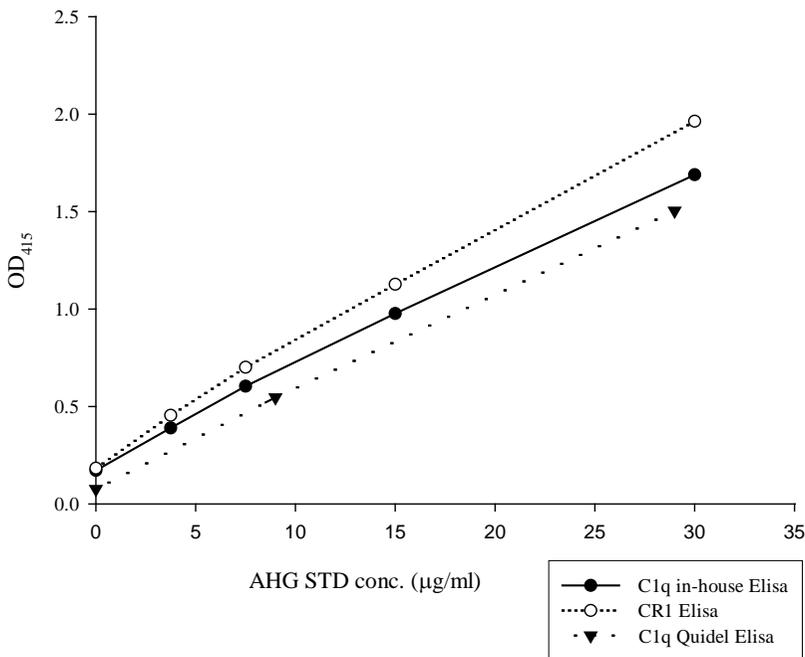

Figure 2: Standard curves generated by C1q Quidel, C1q in-house and CR1 binding assays using AHG dilutions.

CR1 binding inhibition tests

Heat-inactivating normal human serum eliminated CR1 binding completely (Figure 3). This is attributable to the fact that heating at 56°C for 30 minutes inactivates complement proteins and enzymes. We employed this to confirm that CR1 binding assay is actually based on complement activation and requires conversion of C3 to C3b, which will coat the Immune complexes (here it coats the AHG which simulates the IC). This was also set to confirm the specificity of the assay for the signal being observed is attributable mainly to CR1-C3b.
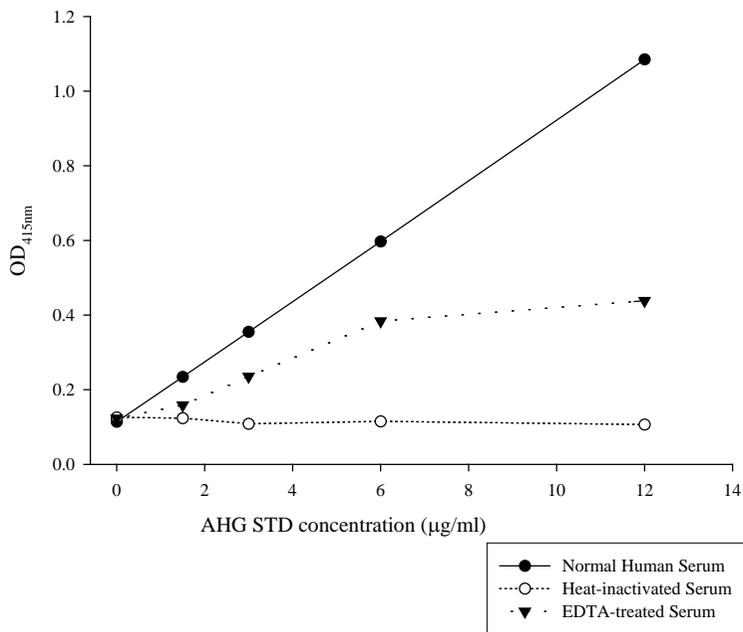

Figure 3: Figure showing the curves obtained when Normal Human Serum, Heat-inactivated Serum and EDTA-treated Serum were incubated with AHG standards.

EDTA treatment reduced the signal significantly. EDTA is a known chelating agent and we used it to demonstrate that CR1-C3b binding requires formation of C3 convertase, which will give rise to cleavage of C3 to get C3b either in the classical or the alternative pathways of complement activation, on which our assay is based. EDTA acts on the bivalent cations (Ca^{2+}, Mg^{2+}), which are involved in proteolytic cleavage of various complement proteins. It also inhibits enzyme formation.

CIC measurement

To quantify CIC, a total of 74 study participants were assessed. They comprised 28 severe malarial anaemia cases, 26 uncomplicated malaria controls and 20 asymptomatic malaria controls. All the three assays showed the same trend in their detection. They all discriminated between the cases and their controls in terms of the level of CIC in their sera (Figures 4 and 5). The commercial Quidel C1q kit showed a significance difference between SA and UM ($p = 0.04$) while C1q in-house assay showed a highly significance difference for the two groups ($p = 0.01$). CR1 binding assay also showed a significance difference between SA and UM ($p = 0.04$). All the three methods showed that there was no significance difference between the two sets of controls, UM and AC. When the cases were compared to the asymptomatic controls, all the three methods showed a significance difference. Quidel kit had $p = 0.001$, C1Q in-house assay had $p = 0.006$ while CR1 binding assay had $p = 0.01$. The level of detection of CIC by CR1 binding assay was comparatively lower compared to C1q binding assay for CR1 binds only ICs that fixes complement.
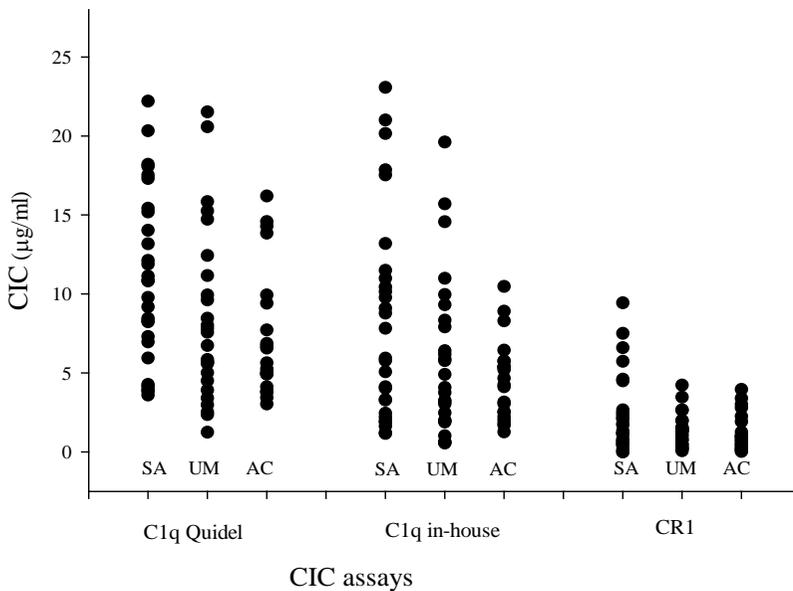

Figure 4: Scatter plots showing the level of Circulating immune Complexes detected by C1q Quidel, C1q in-house and CR1 Binding Assays for severe anaemia (SA) patients and their uncomplicated malaria (UM) and asymptomatic (AC) controls. The dots represent the various study participants for each study group.
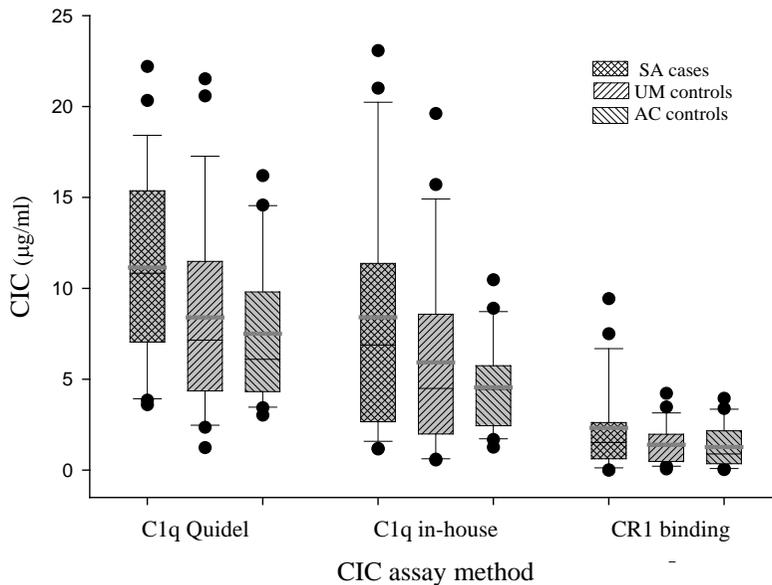
Figure 5: Box- and whisker plots showing the level of Circulating Immune Complexes (CIC) detected by C1q Quidel, C1q in-house and CR1 Binding Assays for severe anaemia (SA) patients and their uncomplicated malaria (UM) and asymptomatic (AC) controls. The box represents the interquartile range and the central 50% of the data values fall within the range of the box, the black horizontal line in the middle of the box marks the median while the gray horizontal line represents the mean of the data. The whiskers are the vertical lines extending up and down from each box and represents the upper and the lower 25% of data, respectively.

Discussion

This study investigated the relationship between circulating immune complexes (CICs), and severe *P. falciparum* malarial anaemia (SA). CICs were quantified using C1q and CR1 binding assays. The data generated from the two methods shows that serum of *P. falciparum* malaria patients with SA contains higher levels of CIC than their respective age and gender-matched controls. C1q binding assay detected higher levels of CIC compared to CR1 Elisa. This can be said to be due to the detection nature of C1q method, it detects a wide range of CIC, and its specificity has been shown to be higher (Stanilova and Slavov, 2001, Mitchel *et al.,* 1987). C1q was chosen because it is the most widely used antigen non-specific CIC quantitative assessment assay hence its properties, like its specificity, have been widely studied. It thus formed the best reference for our novel assay.

C1q and CR1 assays both showed the same trend of detection but the level of CIC detected by CR1 Elisa was comparatively lower than that detected by C1q and this could be attributed to the smaller fraction of CIC

detected by CR1 assay, those that fix complement or the opsonized CIC only. This can also be explained by looking at the properties of the complement proteins detected by CR1 assay, C3b and C4b, they are short lived in circulation. C3b is inactivated by factor I to inactivated C3b (C3bi) (which binds weakly to CR1), and then to C3dg, which is the final degradation product (Kazatchkine and Fearon, 1990, Medof *et al*., 1982). This means that an IC opsonized with C3b first binds to a cell bearing CR1 and at a later stage is released due to C3b degradation (Pascual and Shifferli, 1995, Davies 1990). This has got an implication for CR1 binding assay; it has to be performed as soon as possible after the sample has been obtained in order to capture a higher range of complement fixing ICs when C3b is still intact. Incubation periods should also be made shorter to minimize the risk of further complement activation. CR1 also binds C4b and to a lesser extend C3bi, the immediate degradation product of C3b, but has no affinity for C3dg (Pascual and Shifferli, 1995).

The choice of detection antibody was carefully done to minimize instances of false positive results for it only targets the human antibody constituting the immune complex, and not the free C3b or C4b or C3bi. So the risk of targeting these free or complexed complement proteins has been minimized hence specificity improved, but in spite of this there lies a possibility of modifying this method to use it as a measure of complement activation by targeting C3b/C4b/C3bi, either free or complexed, by having a primary antibody which targets these complement proteins then detecting with the appropriate detection antibody.

In this study, the findings shows elevated levels of CIC in severe malarial anaemia cases, and this has been an area of intense research and has generated a lot of interest as to the link between generation of ICs, hemolysis and its role in pathogenesis of malarial anaemia (Waitumbi *et al*., 2000, Mohamed 1982, Jhaveri *et al*., 1997, Stoute *et al*., 2003, Shepherd *et al*., 1982, Adam *et al*., 1981, Mibei *et al.,* 2008).

Conclusion

CR1 binding assay presents a possibility of assessing a disease associated with intense complement activation for it targets that portion of ICs which fix complement and this could be of importance in management of such diseases as auto immune diseases and even malaria. The use of CR1 binding assay as a method of measuring CIC is highly appropriate in diseases known to be associated with complement activation and disease pathogenesis is known to involve immune adherence, hemolysis or erythrophagocytosis like *P. falciparum* malaria for it gives the actual picture of the CIC fraction which could be responsible for disease progression.

References:

Adam, C.; Geniteuau, M.; Gugerot-Plidalo, M.; Verroust, V.; Lebras, J.; Gilbert, C. and Morel- Maroger, L, (1981). Cryoglobulins, circulating immune complexes and complement activation in cerebral malaria. *Infect. Immun*. 31: 530-535.

Ahearn JM, Fearon DT (1989). Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21). *Adv Immunol*. 46:183-219

Birmingham DJ (1995). Erythrocyte complement receptors. *Crit Rev Immunol.* 15(2): 133-54.

Cornacoff, J.B., Herbert, L., Smead, W., Van Aman, Birmingham, M. and Waxman, F, (1983). Primate erythrocyte immune complex clearing mechanism. J. *Clin. Invest*. 71: 236.

Cosio, FG; Xiao-Ping, S; Birmingham, D; Vantman, M; and Hebert, L, (1990). Evaluation of the mechanisms responsible for the reduction in erythrocyte complement receptors when immune complexes form *in vivo* in primates. *J Immunol* 145:4198-4206.

Davies, KA; Hird, V; Stewart, S; Sivolapenko, GB; Jose, P; Epenetos, A and Walport, M, (1990). A study of in vivo immune complex formation and clearance in man. *J Immunol* 144:4613-4620.

Jhaveri, K., Ghosh, K., Mohanty, D., Parmar, P., Surati, R., Comuens, T., Joshi, H., Iyer, Y., Desai, A. and Badavere, S, (1997). Autoantibodies, immunoglobulins, complement and circulating immune complexes in acute malaria. *The Nat. Med. J. of India*. 10: 5-7

Jones, D**,** Goulding, N., Casey, R. and Gallagher, P. (1982). C1q binding and Raji immune complex assays: a comparison using defined immunoglobulin aggregates. *J. Immunol. Met*. **53**: 201-208.

Kazatchkine MD, Fearon DT (1990). Deficiencies of human C3 complement receptors type 1 (CR1, CD35) and type 2 (CR2, CD21). *Immunodefic Rev.* 2(1):17-41.

Medof ME, Iida K, Mold C, Nussenzweig V (1982). Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes. *J Exp Med*. 156(6):1739-54.

Mibei EK, Otieno WO, Orago AS, Stoute JA. Distinct pattern of class and subclass antibodies in immune complexes of children with cerebral malaria and severe malarial anaemia. Parasite Immunol. 2008 Jun-Jul; 30(6-7):334-41. Epub 2008 May 6

Mibei, EK; Orago, AS; Stoute, JA. Immune complex levels in children with severe *Plasmodium falciparum* malaria. *Am J Trop Med Hyg* **2005**; 72(5):593-599.

Mitchell, J., Batchelor, J., Chapel, H., Spiers, C. and Sim, E. (1987). Erythrocyte complement receptor type 1 (CR1) expression and circulating immune complex (CIC) levels in Hydralazine-induced SLE. *Clin. Exp. Immunol.* **68**: 446-456.

Miyaike J, Iwasaki Y, Takahashi A, Shimomura H, Taniguchi H, Koide N, Matsuura K, Ogura T, Tobe K, Tsuji T (2002). Regulation of circulating immune complexes by complement receptor type 1 on erythrocytes in chronic viral liver diseases. *Gut*. 51(4): 591-6.

Mohammed, I, (1982). The role of immune complexes in human malaria and some of its complications. *J. Infec*. 4: 97-104.

Pascual, M; and Schifferli, JA, (1995). Another function of erythrocytes: Transport of circulating immune complexes. *Infusionsther Transfussionmed* 22:310-315.

Shepherd, S., Burke, P., Thomas, A., Mitchell, G. and Cohen, S, (1982). Circulating immune complexes in *P. knowlesi* infected Kra, and Merozoite vaccinated Rhesus monkeys. *Clin.Exp. Immunol*. 48: 315-320.

Stanilova SA, Slavov ES (2001). Comparative study of circulating immune complexes quantity detection by three assays--CIF-ELISA, C1q-ELISA and anti-C3 ELISA. *J Immunol Methods*. 253(1-2):13-21.

Stoute, JA, Odindo, AO, Owuor, BO, Mibei, EK, Opollo, MO, Waitumbi, JN, (2003). Loss of red blood cell-complement regulatory proteins and increased levels of circulating immune complexes are associated with severe malarial anaemia. *J infect Dis*. 187(3):522-525

Theofilopoulus, A. N, (1980). Evaluation and clinical significance of circulating immune complexes. *Prog. Clin. Immunol*. 4: 63-80.

Waitumbi, J., Opollo, M., Muga, R., Misore, A. and Stoute, J, (2000). Red cell surface changes and erythrophagosytosis in children with severe Plasmodium falciparum anaemia. *Blood*, 95: 1484-1485.

Woodroffe AJ, Border WA, Theofilopoulos AN, Gotze O, Glassock RJ, Dixon FJ, Wilson CB (1977). Detection of circulating immune complexes in patients with glomerulonephritis. *Kidney Int*. 12(4):268-78