Identification and glycobiological characterization of circulating immune complexes in patients with visceral leishmaniasis and post kala azar dermal leishmaniasis

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Here, we investigated the quantitative and qualitative differences in antibody classes and subclasses in serum immune complexes (ICs) of Visceral Leishmaniasis (VL), Post Kala-azar Dermal Leishmaniasis (PKDL) and different cross reactive diseases like Malaria, Leprosy, Vitiligo as compared to control subjects. IC levels were measured through a newly developed PEG ELISA, using L. donovani promastigote membrane antigen coated plate. Antibody classes and subclasses were identified using polyspecific sera and monoclonal antibodies, respectively. ICs were purified using polyethylene glycol (PEG) precipitation. Conditional logistic regression showed an association between IgG1-containing ICs and increased risk of PKDL (OR=75, \(p<0.05\)) and an association of IgG-containing ICs with VL (OR=621, \(p=0.001\)). PEG ELISA demonstrated almost 13-15 fold higher IgG containing ICs titers in VL as compared to control (\(p<0.001\)). The assay further established a significant (\(p<0.05\)) difference in the IgG containing ICs titers between VL and PKDL. The isolated ICs were further analyzed by subjecting them to one-dimensional PAGE and subsequently stained with combination of periodic acid schiff (PAS) with silver. A differential banding pattern between VL and PKDL was obtained. Four distinct bands with carbohydrate rich glycoconjugates were identified in PKDL ICs, which were absent in VL and control group. It suggests the scope for developing a novel differential diagnostic assay.

Keywords: Black fever, Leishmania donovani, PEG-ELISA, Periodic acid schiff staining, Polyethylene glycol,

Leishmaniasis is prevalent in 98 countries worldwide and is one of the most neglected tropical diseases of the world. Third world countries like Sudan, India, Bangladesh, Nepal and Brazil accounts for 90% of visceral leishmaniasis cases till date. In India, Bihar alone contributes to 90% of leishmaniasis cases, which consists of mainly two clinical forms, Visceral Leishmaniasis (VL) and Post Kala-azar Dermal Leishmaniasis (PKDL). An intramacrophage protozoan parasite Leishmania donovani, causes both the diseases, one in the visceral parts residing in macrophages of reticuloendothelial system and the other in peripheral parts encompassing dermal layers of the skin. Leishmania infection leads to the development of antibody specific to the pathogen. The high level of antibodies may drive the formation of immune complexes (ICs), which can bind to the Fc receptors on macrophages, leading to IL-10 production by macrophages, and thus contribute to Leishmania triggered pathogenesis. Consequently, immune complexes can lead to activation of complement system and may deposit in tissues, where it can stimulate an inflammatory response. Immune complexes have been reported to be associated with diseases like SLE, Tuberculosis, Cancers and Rheumatoid Arthritis. In granulomas, extravascular immune complexes play a major role in mycobacterium clearances and subsequent reduction of the bacterial load. Immune complexes in American Visceral Leishmaniasis contain a disease specific 70 kDa Leishmania antigen that possess diagnostic potential. Identification of such novel diagnostic markers in Circulatory Immune Complexes (CICs) has lately generated much interest as they help to design novel diagnostic assays. Till date, there have been only few literature characterizing these CICs in Indian VL. Additionally, the identification and characterization of CICs in Indian PKDL currently remains unaddressed. Thus, characterization of CICs in VL and PKDL could help in the identification of differential disease specific marker, which could help in the development of a differential serodiagnostic assay.
At present, limited serodiagnostic assay which promise differential diagnosis of active VL, cured VL and PKDL have been reported\textsuperscript{12}. On the other hand, there are considerable reports pertaining to identification, characterization of novel diagnostic glycoconjugates in VL patients\textsuperscript{13-16}. Glycoconjugates are disease specific moieties that have been exploited to develop novel diagnostic assays with improved sensitivity and specificity\textsuperscript{17}. However, identification of glycoconjugates in the serum CICs of VL and PKDL patients remains unaddressed. Accordingly, the present study was undertaken to determine the status of CIC bound antibody titers in VL and PKDL patients, so as to develop an improved differential serodiagnostic assay.

**Material and Methods**

*Data collection*—The study was carried out in a government run tertiary care hospital, Calcutta School of Tropical Medicine (CSTM) during July 2013 to June 2014. The study was approved by the Institutional Ethics Committee. Study population consisted of patients with confirmed VL (n=24), PKDL (n=5), Cured VL (n=3) and Cured PKDL (n=2) and Healthy Controls (HC) (n=13). Disease controls (DC) (n=15) included subjects with confirmed Malaria, Multibacillary Borderline (MBT) and Lepramatous Leprosy (LL), Vitiligo and Tinea versicolor. Informed consent was obtained from the enrolled subjects prior to drawing of peripheral blood. The VL was diagnosed by microscopic observation of amastigotes in bone marrow or splenic aspirates as per WHO recommendations (http://www.who.int/tdr/research/progress/leish_prd/summary.html). PKDL cases were either admitted patients or OPD of the department of Dermatology, CSTM. PKDL was diagnosed by clinical features and a history of VL and was corroborated by rK39 strip test, ELISA for anti-leishmanial antibodies, and the presence of Leishman-Donovan bodies in dermal lesions\textsuperscript{18,19}. Sera obtained from the study subjects were aliquoted and stored at −20 °C until further use.

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**PEG Index**—CIC in patient sera were centrifuged at 9055 g for 5 min to remove suspended particles. Then 150 µL of 8% PEG 6000 in BBS, pH 8.4 was mixed with equal volume of the sera and kept overnight at 4 °C. Next day, the pellet was centrifuged at 12,225 g for 10 min to remove the supernatant followed by subsequent washing with 3.5% PEG and reconstituted in 0.01 M PBS and made to a volume of 150 µL. The isolated CIC from all the enrolled study subjects (n=62) was subsequently stored at −20 °C until further use.

**PEG ELISA**—Polystyrene ELISA plates (96 wells, NuncMaxisorp, flat bottom) were coated with optimal parasite antigen concentration (1 µg/100 µL) in PB buffer, pH 7.2 and incubated overnight at 4 °C. The plates were then washed 4 times with PBS buffer containing 0.1% Tween 20 and further incubated at 37 °C with the appropriate dilution of the PEG CIC precipitates (1/50 dilution) for 1 h.

After washing, the plates were incubated with peroxidase conjugated mouse anti human IgG (1:15000) or IgG1 or IgG2 or IgG3 or IgG4 (1:1000) or IgM (1:10000) for 30 min at 37 °C. Finally, the plates were washed and the reaction was developed using Tetramethylbenzediene (TMB) as substrate. Subsequently, the reaction was stopped with 50 µL of 2N H\textsubscript{2}SO\textsubscript{4} and the absorbance was read in microplate reader at 450 nm\textsuperscript{21}. All the tests were performed in triplicate.

**Characterization of circulatory immune complexes**—Isolated CICs (7000 ng) from different patients were analyzed using SDS gel electrophoresis (Laemmli, 1970) and subsequently...
stained with silver staining according to the method of Merrill and Harrington (1984). Further, gels were also subjected to combination of PAS and Silver staining to identify disease specific glycoproteins, if any. Briefly, after the run, gel was fixed with 10% (v/v) trichloroacetic acid (TCA) in water and washed, and subsequently oxidized with periodic acid solution for 40-45 min at RT. Further, it was given a mild acetic acid wash followed by water wash and subsequent treatment with Schiff’s fuschin and sodium metabisulfite reduction while agitating till the band turns magenta. Silver staining was done by subsequent wash of the PAS stained gel with acetic acid and water and dichromate fixation followed by silver nitrate treatment to develop the gel with sodium carbonate in formaldehyde solution for 40-45 minutes. The combined PAS and silver staining technique enabled the visualization of intensified disease specific glycoprotein bands.

**Design and analysis**—Results are presented as mean±S.E.M. by measuring three individual replicates. Statistical analysis was performed using the Graph-Pad Prism statistics software (Graph-Pad Software Inc., San Diego, CA, USA) and between-group comparisons were performed by ANOVA. Differences with \( P < 0.05 \) were considered statistically significant. Diagnostic Odds Ratio analysis was performed using Meta-DiSc version 1.4 software.

**Results**

**PEG Index**—The PEG Index was estimated by the modified Creighton method. The mean±S.E.M. of CIC of VL and PKDL was higher (201.9±14 and 154.27±09) as compared to 127.38±08 for DC and 102±11 for HC as controls (Fig. 1).

**CIC bound antibody levels between cases and controls**—CIC bound antibodies of class IgG, IgM and IgG subclasses were measured among VL and PKDL cases as compared to HC and DC controls.

The mean±S.E.M. of CIC bound IgG levels in VL patients (1.737±0.11) was 13-fold higher than HC (0.137±0.03) and 17-fold higher than DC (0.105±0.01). Similarly, the mean±S.E.M. of CIC bound IgG levels in PKDL patients (0.769±0.20) was 6-fold higher than HC and 7-fold higher than DC. The CIC bound IgG levels between VL and PKDL patients was also significantly different (\( P < 0.001 \)).

Similarly, CIC bound IgG1 levels showed similar trend, where patients with VL and PKDL showed increased levels as compared to HC (0.119±0.01) and DC (0.097±0.01) with significant \( P \) value (<0.05).

No significant differences of CIC bound IgG2 and IgG3 levels were observed in VL and PKDL as compared to HC and DC. Similarly, minimal CIC bound IgG4 levels were obtained in patients with PKDL, VL as well as controls (Table1).

Whereas, slight increase in CIC bound IgM levels were observed in patients with VL (0.156±0.01) as compared to all other groups including PKDL (0.063±0.01), HC (0.091±0.01) and DC (0.041±0.01) (Fig. 2). Longitudinal monitoring of CIC bound antibodies in VL and PKDL patients showed similar values as obtained for HC, further specifying the disease specific nature of CICs (data not shown).

**Table 1**—Determination of isotype and IgG subclasses of PEG precipitated circulating immune complexes in patients with diagnosed VL, PKDL, Healthy Control (HC) and non leishmaniasis disease like Malaria, Leprosy, Vitiligo, Tinea versicolor as Disease Control (DC)

<table>
<thead>
<tr>
<th>IC bound Isotypes and subclasses in PEG ELISA</th>
<th>VL (O.D±SEM)</th>
<th>PKDL (O.D±SEM)</th>
<th>HC (O.D±SEM)</th>
<th>DC (O.D±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>1.737±0.11</td>
<td>0.769±0.20</td>
<td>0.137±0.03</td>
<td>0.105±0.01</td>
</tr>
<tr>
<td>IgG1</td>
<td>0.324±0.05</td>
<td>0.227±0.06</td>
<td>0.119±0.01</td>
<td>0.097±0.01</td>
</tr>
<tr>
<td>IgG2</td>
<td>0.436±0.05</td>
<td>0.394±0.07</td>
<td>0.476±0.02</td>
<td>0.321±0.02</td>
</tr>
<tr>
<td>IgG3</td>
<td>0.694±0.06</td>
<td>0.555±0.05</td>
<td>0.707±0.04</td>
<td>0.376±0.05</td>
</tr>
<tr>
<td>IgG4</td>
<td>0.142±0.01</td>
<td>0.123±0.01</td>
<td>0.144±0.01</td>
<td>0.109±0.01</td>
</tr>
<tr>
<td>IgM</td>
<td>0.156±0.01</td>
<td>0.063±0.01</td>
<td>0.091±0.01</td>
<td>0.041±0.01</td>
</tr>
</tbody>
</table>

Fig. 1—PEG Index comparison of PEG precipitated CICs in study subjects including patients with diagnosed VL cases, PKDL cases, Healthy Control (HC) and non leishmaniasis diseases like Malaria, Leprosy, Vitiligo, Tinea versicolor as Disease Control (DC)
Comparison of CIC levels between VL and PKDL—Conditional logistic regression analysis was performed to identify the class and subclasses of antibody bound CICs that were strongly associated with VL or PKDL cases (Table 2). The association of IgG bound to CICs in patients with VL depicted better OR (OR=621; P <0.001) value than in patients with PKDL (OR=35; P, 0.006). The Odds Ratio (OR) is more favored for IgG1 association to CICs in patients with PKDL (OR=75; P, 0.012) than in patients with VL (OR=57; P, 0.006) (Fig. 3). Thus, among the subclasses, IgG1 bound CICs had significant discriminatory diagnostic potential.

Diagnostic efficacy of PEG ELISA—Comparison of PEG ELISA with serum based ELISA for antileishmanial IgG antibodies revealed that when the cutoff point of both PEG ELISA and serum based ELISA was varied on the basis of the ROC curve, a clear discrimination was evident, showing better efficacy of PEG ELISA (Fig. 4). CIC bound antibodies of class IgG and the subclasses (IgG1, IgG2, IgG3, and IgG4) were determined against the total *L. donovani* antigens. In addition to IgG isotypes, IgM antibodies were analyzed in the CICs, high association of CIC-bound IgG and IgG1 antibodies was observed in both VL and PKDL cases.

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**Table 2—Summary values of Odds Ratio of all the PEG ELISA for VL and PKDL.**

<table>
<thead>
<tr>
<th>CIC bound Antibody Isotypes for VL</th>
<th>Odds Ratio</th>
<th>CI (95%)</th>
<th>P-Value</th>
<th>CIC bound Antibody Isotypes for PKDL</th>
<th>Odds Ratio</th>
<th>CI (95%)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>621</td>
<td>36.75-10492</td>
<td>&lt;0.001</td>
<td>IgG</td>
<td>35</td>
<td>1.34-911</td>
<td>0.0325</td>
</tr>
<tr>
<td>IgG1</td>
<td>57</td>
<td>3.124-1039</td>
<td>0.0064</td>
<td>IgG1</td>
<td>75</td>
<td>2.56-2196</td>
<td>0.0122</td>
</tr>
<tr>
<td>IgG2</td>
<td>24.42</td>
<td>1.31-454</td>
<td>0.0322</td>
<td>IgG2</td>
<td>2.27</td>
<td>0.039-129</td>
<td>0.6909</td>
</tr>
<tr>
<td>IgG3</td>
<td>29.36</td>
<td>1.59-542</td>
<td>0.0231</td>
<td>IgG3</td>
<td>1.727</td>
<td>0.0298-99</td>
<td>0.7918</td>
</tr>
<tr>
<td>IgG4</td>
<td>20.02</td>
<td>1.06-377</td>
<td>0.045</td>
<td>IgG4</td>
<td>2.636</td>
<td>0.0463-149</td>
<td>0.6382</td>
</tr>
<tr>
<td>IgM</td>
<td>41.27</td>
<td>2.25-755</td>
<td>0.0121</td>
<td>IgM</td>
<td>1.909</td>
<td>0.0331-109</td>
<td>0.7545</td>
</tr>
</tbody>
</table>

Comparison of CIC levels between VL and PKDL—Conditional logistic regression analysis was performed to identify the class and subclasses of antibody bound CICs that were strongly associated with VL or PKDL cases (Table 2). The association of IgG bound to CICs in patients with VL depicted better OR (OR=621; P <0.001) value than in patients with PKDL (OR=35; P, 0.006). The Odds Ratio (OR) is more favored for IgG1 association to CICs in patients with PKDL (OR=75; P, 0.012) than in patients with VL (OR=57; P, 0.006) (Fig. 3). Thus, among the subclasses, IgG1 bound CICs had significant discriminatory diagnostic potential.
However, as shown in Table 3, IgG2, IgG3, IgG4 and IgM were unable to establish any positive predictive values in case of CICs of sera with PKDL cases. Very low levels of CIC-bound IgM for VL cases occurred, though PEG IgM ELISA yielded good sensitivity and specificity values as compared to PKDL.

Thus, among the 24 VL cases, 23 (96%) were positive for CIC IgG. Whereas, only 1 (7%) case among DC group was falsely detected for CIC IgG, and HC group showed no detectable IgG CICs. The sensitivity values for PEG IgG were 96% and 60% for VL and PKDL, respectively. Similarly, the sensitivity values for PEG IgG1 were 50 and 80% for VL and PKDL, respectively (Table 3).

The diagnostic test characteristics namely sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated based on the PEG ELISA. The results are listed in Table 3. A wide variation of sensitivity values for different CIC bound IgG subclasses in PEG ELISA were observed for VL (50% to 25%). However, the sensitivity values for the diagnosis of VL cases by CIC bound IgG total was an impressive 95.83, PPV being 95.83 and NPV being 96.43. On the other hand for PKDL, maximum sensitivity was obtained only with CIC bound IgG1 (80%), other values like PPV (100%) and NPV (92.31%) were also high (Table 3).

**SDS PAGE analysis**—The CICs isolated from patients and controls by PEG precipitation were submitted to one dimensional SDS PAGE. They were subsequently processed for silver stain, the gel picture showed a lot of protein bands both major and minor. Differential enhanced banding pattern between VL and PKDL was obtained only when PAS and silver staining technique was applied. This method specifically detects glycosylated protein having sialic acid and other carbohydrates. Nine overlapping bands (40-70 kDa) were obtained in PKDL CICs, whereas one prominent band (46 kDa) was obtained in VL CICs. Molecular weight of each band was

<table>
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<tr>
<th>Table 3—Diagnostic test characteristics of PEG ELISA</th>
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<tbody>
<tr>
<td><strong>PEG ELISA for VL</strong></td>
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<tr>
<td>IgG</td>
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<td></td>
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<tr>
<td>IgG1</td>
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<td>IgG2</td>
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<td>IgG3</td>
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<td>IgG4</td>
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<tr>
<td>IgM</td>
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<tr>
<td><strong>PEG ELISA for PKDL</strong></td>
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<tr>
<td>IgG</td>
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<tr>
<td>IgG1</td>
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<td>IgG3</td>
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<td>IgG4</td>
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<td>IgM</td>
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</table>

(*) Lack of establishment of PPV, where PPV denotes Positive predictive value and NPV denotes Negative predictive value.
subsequently calculated through $R_f$ value generated from best fitted curve of $R_f$ value vs. log of Molecular weight of markers. Analysis revealed that CICs of VL, PKDL, HC and DC had 1 common glycoprotein band (71 kDa). Further, analysis revealed that the CIC of VL had only one exclusive glycoprotein band of 46 kDa, which was absent in CIC of PKDL, HC and DC. Whereas, PKDL CIC contained four glycoprotein bands of approximately 44, 49, 59 and 61 kDa, respectively, which were completely absent in the other categories (Fig. 5). Thus, a characteristic differential banding pattern was obtained between CIC of VL and PKDL patients.

Discussion

The direct demonstration of parasite load by bone marrow smear examination or skin slit smear examination for VL and PKDL are still considered the golden standard of diagnosis for leishmaniasis (http://www.who.int/leishmaniasis/resources/documents/VL_NMR_1107_ok.pdf). Both the methods, however, suffer from the disadvantage of being invasive and painful. Further, as in PKDL patients lesions occur mainly on the face, the demonstration of parasites in skin smears or biopsy specimens are difficult. The disease may also include nerve involvement and other similar factors and, therefore, could be misdiagnosed as leprosy. Similarly, the clinical presentations of VL often pose a diagnostic dilemma for the clinician as well. In the Indian subcontinent, unresolved cases of VL and PKDL are considered to be the reservoir to house and disseminate the causative parasite in spreading leishmaniasis. The early diagnosis of both forms of leishmaniasis in India, remains the biggest challenge for its eradication.

The strong humoral response that usually accompanies leishmanial infections, has enabled for the development of novel serological techniques for the accurate diagnosis of VL. In comparison, attempts for the serodiagnosis of PKDL have been few, and have various random sensitivity and specificity. Anti-leishmanial antibodies of the IgG, IgM, and IgG subclasses have been demonstrated in the sera of PKDL patients through serum based ELISA. However, to our knowledge, determination of the CIC bound subclass distribution of the antibody response for the diagnosis of VL and PKDL has not been investigated.

Accordingly, in this study, we have measured the CIC bound antibodies of class IgG, IgM and IgG subclasses by a PEG ELISA both in VL and PKDL patients and compared them systematically with both normal healthy and disease controls. PEG ELISA when compared with serum based ELISA generated better AUC values through ROC analysis, establishing its superiority over the latter (Fig. 4). More accurately, CIC bound IgG yielded good DOR values and could successfully differentiate PKDL cases from VL cases. Further, when the Cochran Q test was performed, a good homogeneity with significant p-value (0.53) was established (Fig. 3). Thus, the diagnostic characteristics enhance the potentialities of CIC bound antibodies in differential diagnosis as well as overall diagnosis of leishmaniasis.
Moreover, the characterization of glycoprotein components present in the CICs of patients establishes the differential diagnosis of PKDL and VL (Fig. 5).

The CICs containing different types of immunoglobulins and complement components have been demonstrated in leishmaniasis by earlier researchers. In the present study, enhanced PEG Index was also observed both in VL and PKDL, indicating high titers of CICs (Fig. 1). Furthermore, CIC bound IgG as measured by PEG ELISA demonstrated almost 13-15 fold higher values for VL than normal and cross reactive diseases indicative of high immune complexes formation (Fig. 2). CIC bound IgG in PKDL on the other hand had almost 6-7 folds increased OD values than age and sex matched control values. Concomitantly, protein estimation of the patient CICs by Lowry assay also supported the PEG ELISA data (data not shown). Further, analysis revealed that the CIC bound IgG1 could successfully diagnose PKDL cases. The SDS PAGE analysis reinforced the fact that VL and PKDL can be distinguished on the basis of the differential banding pattern of CICs. SDS PAGE analysis identified four glycoprotein components in PKDL CICs, which could be treated as potential candidates for differential serodiagnosis between VL and PKDL.

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Conflict of interest statement
We declare that we have no conflict of interest.

References


