CHAPTER III

HUMORAL RESPONSES IN
INDIAN PKDL
Introduction

Humoral immunity is an effector arm of adaptive immunity that provides defence against any pathogen that invades our body, particularly against extracellular microbes and their toxins. Although complement proteins are included in the humoral branch of immunity, the mainstay of defense generally is antibody mediated. When pathogenic microbes invade the human body, they interact aggressively with constituents of the host immune system and as a rule, are able to downsize almost any cellular, humoral, or systemic immune response to establish themselves within the host body [Kotwal, 1997]. In leishmaniasis, B cells and antibodies are however not considered to be of major importance in protective immunity [Saha et al. 2006]. Antibodies are not effective at eliminating the parasite possibly because it hides within the parasitophorous vacuole of host macrophages [Kaye and Scott, 2011].

Antibody responses in cutaneous leishmaniasis (CL)

In CL, antibody responses are usually lowered during active phase of the disease attributed to the localized nature of the disease [Reviewed in Sharma and Singh, 2009]. In patients with CL, the role of antileishmanial antibodies is as a marker for both diagnostic and prognostic marker [Fagundes-Silva GA et al. 2012, Brito et al. 2001, Valli et al. 1999]. Importantly, in a recent study, Fagundes-Silva GA et al. (2012) showed a decrease in antileishmanial IgG1 and IgG3 after healing of lesions in CL, correlated with the time of clinical cure. In experimental CL, IgG has been demonstrated to be beneficiary for the parasite as IgG coated amastigotes of L. major enter into the host macrophages through Fc receptors, induce IL-10 production thus establish the infection [Kane and Mosser 2001]. In support of this model an in vivo study by Okwor et al. (2009) found that Fc deficient mice infected with L. amazonensis produce less IL-10 and are less susceptible to infection. On the contrary, antileishmanial antibodies, B cells are beneficial in mucocutaneous leishmaniasis (MCL) as uptake of promastigotes through FcγRI on dendritic cells facilitates generation of protective T cell immunity [Scott et al. 1986]. Similarly IgE responses in CL have been shown to be beneficial as ligation of IgE with CD23 on monocyte/macrophage surface potentiates generation of the potent anti-parasitic molecule nitric oxide [Reviewed in Mossalayi et al. 1999].

Antibody responses in visceral leishmaniasis (VL)

Visceral leishmaniasis or VL has always been documented as being an elevated antileishmanial antibody response, although the exact role of this antibody response is unclear [Reviewed in Saha et al. 2006]. Detailed analysis of Leishmania antigen specific immunoglobulin isotypes revealed raised levels of antileishmanial IgM, IgG, IgE and IgG subclasses during disease [Reviewed in Sharma and Singh 2009, Saha et al. 2006]. In VL
antileishmanial antibody levels have been correlated with many parameters including disease chronicity [Redhu et al. 2006], delayed type hypersensitivity [Hailu et al. 2001] and as surrogate markers of cytokine response [Atta et al., 1998] and most importantly, with antimony resistance [Chatterjee et al. 1998, Anam et al. 1999]. In an attempt to associate antibody isotypes with disease progression, Anam et al. (1999a) showed that a decrease in IgG2 and IgG3 levels was linked to antimony refractoriness. Titres of other isotypes and IgG4 showed no difference with regard to antimony unresponsiveness and IgG1 levels remained elevated in all patients [Anam et al., 1999]. On the contrary in another study, antimony unresponsive cases showed unaltered or slightly increased levels of IgG subclasses compared to levels at presentation, additionally, in antimony responsive cases, curtailment of IgG subclasses were observed [Chatterjee et al. 1998]. Taken together, antileishmanial antibody levels shows wide discrepancy of the data between various groups [Ghosh et al. 1995, Chatterjee et al. 1998, Anam et al. 1999, Hailu et al. 2001, Ganguly et al. 2008, Ansari et al. 2008b]; which can be attributable to regional differences, ethnic variations, number of samples, type of antigen used, age of the patients etc.

Moreover, a regulatory role for B cells has been suggested in experimental VL demonstrating that B cell depleted mice exhibit extensive neutrophils mediated pathology [Smelt et al. 2000]. Recently, it was also suggested that marginal zone B cells suppress protective T cell responses in the early stages of experimental VL [Bankoti et al. 2012]. Thus, in an animal model, development of a strong humoral response is more associated with pathology than protection or resolution of disease. However in VL patients, such studies are limited and till date antileishmanial antibody responses has been used for diagnosis [Reviewed in Saha et al. 2006, Chappuis et al. 2007] but found to be limited as a predictive of disease [Gidwani et al. 2009]. It also failed to be effective as a prognostic marker due to persistence of antibodies well after cure [Chappuis et al. 2007, Gidwani et al. 2011].

Status of Humoral immunity in PKDL

Similarly like in VL, antibody responses in PKDL are used for diagnostic purpose although the gold standard method is the skin smear staining but does not give satisfactory result in macular PKDL [Salotra and Singh 2006]. Several studies have shown that patients with Indian PKDL demonstrate increased levels of IgG and its subclasses, but the order of response is varied [Haldar et al. 1981, Ghosh et al. 1995, Saha et al. 2005, Ganguly et al. 2008, Ansari et al. 2008b]. The large discrepancy of results is either due to the nature of the antigen used for ELISA or the study population, which is always a major limitation for studying Indian PKDL. Another major problem arises when one compares the levels of IgG4 and IgE when it was reported to be absent in PKDL [Saha et al. 2005] and elevated in another [Ansari et al. 2008b]. Furthermore, till date no study has been conducted to assess the
humoral response in different clinical variants of PKDL (polymorphic and macular) as well as the effect of different antileishmanials that are used for treating the patients. Importantly detailed information of the humoral immune picture in Indian PKDL in a larger study cohort was necessary which this study aimed to achieve.

**Study objectives**

With a view towards identifying a potential serological marker for addressing the above lacunae, the aim of this study was to dissect the humoral response in macular and polymorphic PKDL in terms of antileishmanial Ig, IgG and their subclasses, IgM and IgE status as also assess the differential antibody response after treating patients with either sodium antimony gluconate (SAG) or Miltefosine.

**Materials and Methods**

**Preparation of Leishmania donovani antigen (LDA)**

Crude *Leishmania* antigen was prepared from a *L. donovani* strain, MHOM/IN/90/GE1F8R as detailed in Materials and Methods (chapter 2).

**ELISA for antileishmanial Ig, IgM, IgG and IgE**

Total anti-leishmanial Ig, IgM, IgG, IgE binding to LDA were detected using protein A conjugated horseradish peroxidase (HRP), anti human HRP-IgM, anti human HRP-IgG, anti human IgE conjugated with alkaline phosphatase respectively as described in Materials and Methods (chapter 2).

**Subclass ELISA**

Anti-leishmanial IgG subclass distribution was determined by subclass ELISA using either unconjugated anti-human IgG1, biotinylated anti-human IgG2, IgG3 or IgG4 and binding detected with anti-mouse Ig-HRP or Streptavidin-HRP, as described in Materials and Methods (chapter 2).

**Immunophenotyping of peripheral blood leukocytes**

Peripheral blood (100 μl) was surface stained with fluorochrome conjugated antibodies to CD3 Peridinin chlorophyll protein or PerCP, CD19 Allophycocyanin or APC as described in materials and methods (chapter 2).

**Determination of IgG avidity**

IgG avidity was measured using urea to weaken the binding between antigen and antibody as described in Materials and Methods (chapter 2).
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Results

Elevated levels of antileishmanial Ig, IgG and IgE in patients with PKDL

Patients with PKDL (polymorphic = 41, macular = 16) were analyzed for measurement of antileishmanial Ig, IgM, IgG and IgE (Figure 3.1) and compared with healthy controls (n = 15) along with post treatment (n = 28). Total antileishmanial Ig levels in PKDL patients was a significant 8.17 fold higher than healthy controls, the median (IQ range) of O.D$_{405}$ being 0.49 (0.27-0.8) vs. 0.06 (0.02-0.15) respectively (Figure 3.1A, p<0.0001) and remained 6.33 fold higher following treatment 0.38 (0.20-0.52, Figure 3.1A, p<0.0001). IgM levels remains unaltered in patients both pre and post treatment when compared to healthy controls, median (IQ range) of O.D$_{405}$ being 0.16 (0.11-0.30) and 0.14 (0.10-0.23) vs. 0.16 (0.07-0.20) respectively (Figure 3.1B). IgG was the major immunoglobulin raised in patients with PKDL. We found a significant 3.75 fold increase in levels of IgG in patients compared to healthy controls, median (IQ range) of O.D$_{405}$ being 0.45 (0.25-0.86) vs. 0.12 (0.03-0.15) respectively (Figure 3.1C, p<0.0001) which again remained unaltered following treatment 0.40 (0.23-0.63, Figure 1C, p<0.0001). IgE levels, although a relatively minor contributor to the elevated humoral response in PKDL was found to be significantly raised in patients with PKDL (Figure 3.1D, p<0.05), median (IQ range) of O.D$_{405}$ being 0.01 (0.00-0.08) vs. 0.00 respectively. Treatment caused no change in IgE levels, 0.01 (0.00-0.04).

Elevated levels of Ig in both polymorphic and macular PKDL

Higher levels of antileishmanial immunoglobulin (Ig) were detected in both polymorphic and macular PKDL (Figure 3.2A), with the polymorphic group being 8.4 fold
and the macular group 4.3 fold higher than healthy controls (Figure 3.2A). Furthermore, the Ig levels in the polymorphic group was significantly higher than the macular group, the median (IQ range) of O.D\textsubscript{405} being 0.60 (0.36-0.93) vs. 0.31 (0.20-0.45) respectively (p<0.05, Figure 3.2A). This indicated that in polymorphic PKDL, the humoral response was stronger than in the macular variant, possibly attributable to the relatively longer duration of disease along with a higher antigen load. Upon treatment, polymorphic patients showed a dramatic regression in levels of Ig (p<0.0001, Figure 3.2B), but a similar decrease was notably absent in the macular group (Figure 3.2B).

IgG was the major contributor towards the elevated Ig response in polymorphic PKDL

The levels of IgM, IgG and IgE as determined by ELISA indicated that IgM was not significantly elevated in patients with PKDL as compared to healthy controls, irrespective of the clinical variant (Figure 3.2C) and is not surprising, considering the chronicity of the disease. Treatment caused a significant decrease in levels of IgM in patients with polymorphic lesions (p<0.05, Figure 3.2D), but not in patients with macular lesions (Figure 3.2D). In human leishmaniasis, increased levels of IgG have been consistently reported [Sharma and Singh 2009] and similarly in PKDL, we observed that levels of IgG were significantly elevated in both polymorphic [0.62 (0.27-0.92), p<0.001] and macular PKDL [0.36 (0.17-0.56), p<0.05] as compared to healthy controls [0.12 (0.03-0.15), Figure 3.2E]. The elevation of IgG was more prominent in the polymorphic group, owing to the longer duration of disease and possibly disease severity. In the macular type, the IgG levels were 1.46 fold lower than the polymorphic variant. Treatment translated into a significant decrease of IgG in the polymorphic group (p<0.01, Figure 3.2F), whereas macular PKDL patients showed only a marginal decrease (Figure 3.2F). In PKDL, only one study has been done with 23 patients where the absence of Leishmania antigen specific IgE was reported [Saha et al. 2005]. However, in our study, with a larger study population, levels of IgE although far lower than IgG and IgM were significantly raised in both polymorphic [0.01 (0.00-0.09), p< 0.05] and macular PKDL [0.02 (0.00-0.13), p< 0.05] as compared to healthy individuals, who had near non-detectable levels of IgE [0.00 (0.00-0.01), Figure 3.2G]. As levels of IgE were very low, with large inter-individual variations, the effect of treatment on curtailment of IgE was not evident (Figure 3.2H).
Miltefosine is a greater modulator of the humoral immune response in PKDL

Miltefosine has been proposed to exert its effect through a direct anti-parasitic and indirect immunomodulatory activity. On the other hand, its function was also found to be immune independent as in SCID mice, its effectiveness remains unaltered, whereas the action of SAG action was immune dependent. However, studies in human leishmaniasis are scanty and in this study, we found that PKDL patients who received Miltefosine has significantly lowered levels of Ig, IgM, IgG following treatment vis a vis patients who received SAG treatment has only decreased levels of Ig and IgG level (Figures 3.3A-C). Importantly, IgE which was shown to have antileishmanial effect via ligation with CD23, is increased significantly with Miltefosine treatment but, not with SAG (p<0.05, Figure 3.3D).
Distribution of IgG subclass in patients with PKDL

Among the IgG subclasses, IgG1 and IgG3 levels were significantly higher than healthy controls [0.36 (0.17-0.76) vs. 0.03 (0.01-0.08) and 0.61 (0.19-1.51) vs. 0.16 (0.14-0.19), p<0.0001, Figures 3.4A and C respectively]. Both IgG1 and IgG3 remained significantly raised even following treatment [0.26 (0.04-0.59) and 0.60 (0.20-0.76), p<0.01 and p<0.05, Figures 3.4C and D respectively]. Regarding IgG2 or IgG4, no change was observed at presentation or after treatment when compared to healthy controls [0.09 (0.02-0.17) vs. 0.05 (0.02-0.13) vs. 0.15 (0.06-0.29) and 0.13 (0.02-0.24) vs. 0.06 (0.04-0.11) vs. 0.13 (0.06-0.35), Figures 3.4B and D respectively].
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Among the subclasses, IgG1 and IgG3 levels in polymorphic PKDL was 14.5 and 4.5 fold higher than healthy controls [0.41 (0.26-0.87) vs. 0.03 (0.01-0.08), p<0.001 and 0.55 (0.20-1.61) vs. 0.16 (0.14-0.19), p<0.01 respectively, Figures 3.5A and 5E]; with treatment, both IgG1 and IgG3 levels in polymorphic patients decreased significantly (p<0.01 and p<0.001; Figures 3.5B and 5F respectively). However, in the macular variant, only IgG1 was 7 fold higher than healthy controls [0.33 (0.12-0.41) vs. 0.03 (0.01-0.08), p<0.05, Figure 3.5A] but not IgG3 (Figure 3.5E); with treatment, neither IgG1 nor IgG3 levels decreased in these patients (Figures 3.5B and 5F respectively). With regard to IgG2 and IgG4, minimal changes were found in both polymorphic [0.08 (0.00-0.16) and 0.13 (0.02-0.26) respectively] and macular [0.11 (0.04-0.22) and 0.13 (0.07-0.21) respectively] groups when compared with healthy subjects [0.05 (0.02-0.13) and 0.06 (0.04-0.11) respectively, Figures 3.5C and 5G]. Although the levels of IgG2 was not increased in polymorphic PKDL, a significant curtailment occurred with treatment (p<0.05, Figure 3.5D); no changes in levels of IgG4 was observed. Following treatment, patients with macular PKDL showed no changes in IgG2 or IgG4 (Figure 3.5H). So the order of IgG subclass levels in polymorphic PKDL is IgG3>>IgG1>>IgG4>>IgG2, while for macular PKDL, it is IgG3>>IgG1>IgG2>IgG4.

Figure 3.4: Scatter distributions of antileishmanial IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D) in healthy controls (n = 15, ●), patients with PKDL at presentation (pre t/t, n = 45, ■) and post treatment (post t/t, n = 22, ▲).

Raised IgG1 and IgG3 in polymorphic PKDL
Effect of Miltefosine and SAG on IgG subclass levels

With regard to IgG subclasses, IgG3 and IgG1 levels (strictly in that order) were elevated in Indian PKDL. Armed with this knowledge, we aimed at analyzing the effects of Miltefosine and SAG on the subclass profile. Interestingly, both IgG1 and IgG3 levels, like total IgG, showed significant reduction after treatment with Miltefosine (p<0.01, Figures 3.6A and C). A similar trend was obtained with SAG although differences were not statistically significant (Figures 3.6A and C). IgG2 and IgG4 levels, as shown earlier, were unchanged (Figures 3.6B and D).

Figure 3.6: Effect of SAG and Miltefosine on antileishmanial IgG subclass levels in patients with PKDL. Before and after plots of, IgG1 (A); IgG2 (B), IgG3 (C) and IgG4 (D), patients before treatment are marked with circles and after treatment with triangles.
Loss of CD19 expression supports the activation of antibody secreting plasma cells in PKDL.

The significantly decreased expression of CD19 in patients with PKDL as compared to healthy controls [9.75 (6.27-13.54) vs. 20.72 (12.48-21.94), p <0.05, Figure 3.7] indicated that in PKDL, B cells were being transformed into antibody secreting plasma cells. Treatment caused a significant increase in CD19 expression [15.43 (12.40-22.73), p<0.05, Figure 3.7]. Both polymorphic and macular PKDL patients had a lowered expression of CD19 [9.56 (6.07-18.04) and 12.49 (6.41-13.41), Figure 3.7.

![Figure 3.7: Comparison of CD19 expression between healthy controls (n = 7, •), patients with PKDL at presentation (Pre t/t, n = 22) and after treatment (Post t/t, n = 12). Similarly between healthy controls (n = 7), polymorphic PKDL (n = 14) and macular PKDL (n = 8).](image)

Raised levels of IgG avidity in polymorphic PKDL

As determination of IgG avidity can help to indicate the time course and duration of disease, we studied this in patients with PKDL, wherein patients with polymorphic lesions had a significantly higher IgG avidity than those with macular lesions [69.00 (57.30-81.41) vs. 54.27 (50.50-64.75), p<0.05, Figure 3.8A]. Furthermore, IgG avidity correlated with duration of disease of polymorphic PKDL (r = 0.42, p<0.05, Figure 3.8B), not with macular PKDL (r = 0.19, Figure 3.8C).
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Discussion

Mice and humans infected by *Leishmania* exhibit high levels of parasite specific IgG but do not provide protective immunity, because these antibodies are not effective at killing parasites that hide inside host macrophages [Mougneau et al. 2011]. Antileishmanial antibodies correlated with disease severity [Castellano et al. 2009]. Similarly in our study, we found that patients with PKDL had an elevated total antileishmanial antibody response in terms of total Ig, IgG and IgE (Figure 3.1) and, the response was elevated in the polymorphic group (Figure 3.2). Importantly, IgM was not significantly elevated in patients with PKDL as compared to healthy controls, irrespective of the clinical variant (Figure 3.1C). A similar finding was reported in Sudan [el Amin et al. 1986] and is not surprising, considering the chronicity of the disease. However, Haldar *et al.* reported increased levels of IgM in Indian patients with PKDL [Haldar *et al.* 1981]. Treatment caused a significant decrease in levels of IgM in patients with polymorphic lesions (Figure 3.2D), but not in patients with macular lesions (Figure 3.2D). This indicated that in polymorphic PKDL, the humoral response was stronger than in the macular variant, possibly attributable to the relatively longer duration of disease or higher antigen load.

The debatable role of antileishmanial Igs has been studied in many experimental models but remains unclear [Kane and Mosser 2001, Miles *et al.* 2005, Vouldoukis *et al.* 1994, Vouldoukis *et al.* 1995]. Passive administration of antileishmanial IgG to BALB/c mice resulted in appearance of larger lesions along with increased amounts of IL-10 [Miles *et al.* 2005]. This was attributed to the binding of IgG to Fc receptors on macrophages and it was proposed that these IgG-opsonised parasites survived better in the host [Kane and Mosser 2001]. It is also possible that IgG is lethal for *Leishmania* promastigotes following activation of the complement pathway [Ellassad *et al.* 1994]. In human leishmaniasis, increased levels of IgG have been consistently reported [Reviewed in Sharma and Singh, 2009] and similarly in PKDL, we observed that levels of IgG were significantly elevated in both polymorphic and macular groups (Figure 3.2E). In leishmaniasis, high levels of IgE have been documented in VL and CL [Atta *et al.* 1998, Sousa-Atta *et al.* 2002]. However, in PKDL, the data was
limited [Saha et al. 2005] and our study showed an increased levels of IgE (Figures 3.1D and 3.2G).

As proliferation and differentiation of B lymphocytes to plasma cells is regulated by T cells and soluble cytokines, measurement of individual IgG subclasses may indirectly reflect polarization of immune responses and thereby serve as surrogate markers of T cell responsiveness. Re-evaluation of the IgG subclasses in a larger study group is needed due to variability of the existing data [Haldar et al. 1981, Ghosh et al. 1995, Saha et al. 2005, Ganguly et al. 2008, Ansari et al. 2008b] and we observed a raised IgG1 and IgG3 in patients. Additionally, our study re-established the order of IgG subclass levels in polymorphic PKDL (IgG3>IgG1>IgG4>IgG2) and for macular PKDL (IgG3>IgG1>IgG2>IgG4, Figures 3.4 and 3.5]. As PKDL is sequelae of VL, the problem of drawing the serological response is to some extent difficult because antibodies can be stable up to many years within the patients who have prior history of VL [Chappuis et al. 2007]. To resolve this problem we tested the IgG avidity and expression of CD19. Herein, we found a decreased CD19 expression on B cells in PKDL which is restored following treatment suggesting that, in PKDL the antileishmanial antibodies are not the preformed from their VL period, and are instead due to a secondary response to parasites (Figure 3.7). Our data suggested that, polymorphic groups whose disease was more chronic have higher IgG avidity than the macular PKDL (Figure 8). Furthermore, correlation with disease duration enforced that chronicity of disease among polymorphic group was higher (Figure 3.8).

Monitoring of the disease status and their response towards antileishmanial chemotherapy is essential for patients with PKDL owing to the fact that these patients are potential reservoirs for VL and elimination of leishmaniasis has been targeted at 2015 [Mondal et al. 2009]. Although several markers have been used for diagnosis of PKDL, none have proved effective and one is still dependent on clinical evaluation. Furthermore, as SAG which is notorious for its high propensity towards unresponsiveness is still used for treatment of PKDL, it emphasises the need for developing prognostic markers. In our effort to assess the immunomodulatory effect of antileishmanials (SAG and Miltefosine) in PKDL patients, we observed that Miltefosine exerted greater effect on reducing the levels of Ig, IgM IgG, IgG1 and IgG3 levels whereas SAG treatment mediated curtailment was evident only in Ig and IgG levels (Figures 3A,B and C and Figure 6). However, one cannot predict whether this curtailment in antileishmanial Ig, IgM and IgG is a prophylactic effect or just a consequence, because, a decreased antibody response after cure was always evident [Reviewed in Saha et al. 2006]. Rather one could predict the immunomodulatory role of either SAG or Miltefosine by assessing IgE levels. IgE has been reported to be beneficial for generation of the anti-parasitic molecule nitric oxide within monocytes/macrophage subsets following ligation with its receptor CD23 [Reviewed in Mossalayi et al. 1999, Vouldoukis et al. 1994]. Miltefosine
significantly increased the level of IgE (Figure 3.3), thus probably causing an increased generation of NO than SAG similar to previous reports [Sarkar et al. 2010]. Taken together, Miltefosine elicited a stronger immunomodulatory effect than SAG in lowering IgM and IgG along with increasing IgE and therefore could be considered as more effective management of PKDL.