CHAPTER 4

ANTIBODY RESPONSES AND CIRCULATORY CYTOKINES IN INDIAN PKDL
Introduction

The magnitude of infection by *Leishmania* depends not only on the causative species but also on the immunological status of the affected individual—whether he or she is able to resist the parasite-mediated onslaught on the body’s defences. However, a single *Leishmania* strain may actually cause more than one disease variant with the host’s immune response also responsible for differences in the form of the disease (Liew and O’Donnell, 1993). 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 (Kotwal, 1997). Strangely, in *Leishmania* infections, moderate to strong humoral responses are the rule rather than the exception with elevated titres of anti-leishmanial antibodies seen. This paradox is explained by the ability of Th2 cytokines like IL-4 to promote antibody synthesis by plasma cells (Mosmann and Coffmann, 1989) together with the high IL-4 levels documented in both clinical and murine forms of leishmaniasis (Kemp et al., 1993a). Yet, this enhanced humoral response confers no benefits on the host and as such, the presence of the antibodies cannot be linked to pathogenesis, either.

Cutaneous leishmaniasis

In the cutaneous form, low levels of anti-leishmanial antibodies are measured typically during the active phase of the disease (Behin and Jacques, 1989). In patients with clinical American cutaneous leishmaniasis, serological analysis with several *L. braziliensis* antigens demonstrated a reduction in IgG reactivity with clinical cure, spontaneous or drug-treated. Accordingly, this study was able to confirm the utility of the dynamics of the antibody response in monitoring clinical cure of cutaneous leishmaniasis (Brito et al., 2001). In mucosal leishmaniasis, the most disfiguring and morbid sequela of cutaneous leishmaniasis caused by *L. braziliensis*, immunoblotting with anti- *L. braziliensis* IgG positive patient sera showed differences in promastigote soluble antigen binding between mucosal and cutaneous leishmaniasis, establishing the prognostic value of the transition from a cutaneous antigenic profile to a mucosal one (Valli et al., 1999). Since timely diagnosis of mucosal leishmaniasis is beset by problems with routine
parasitology, which would otherwise have enabled effective treatment of mucosal leishmaniasis, humoral immune markers are thus, of significant value.

**Visceral leishmaniasis**

The visceral form is characterized by pronounced polyclonal hypergammaglobulinemia. Strangely, though VL patients present with abundant nonspecific immunoglobulin as well as specific antileishmanial antibodies, they fail to respond to parasite antigen in the delayed-type hypersensitivity skin test, an indicator of suppressed cell-mediated immune response (White and McMahon-Pratt, 1988). In stark contrast to the low antibody titres documented in CL, high anti-leishmanial antibody titres are characteristic of VL cases, worldwide (Bray, 1975; Neogy et al., 1987). Often, a temporary humoral response occurs during the active phase of VL with the appearance of low antibody titres that disappears a few months after conclusion of treatment, making serological assessment indispensable for prognosis and also for delimiting the period during which clinicians should observe patients in order to make a prognosis (Behin and Jacques, 1984).

Antileishmanial antibody isotypes (IgG and IgM) and subtypes (IgG1, IgG2, IgG3, and IgG4) were observed in all patients with American visceral leishmaniasis; IgA was detected in fewer patients; treatment reduced antibody levels and IgG4 was found to correlate with the infection sustaining cytokine, IL-4 that was also reduced with cure (da Matta et al., 2000). Further, as IL-4 is also associated with synthesis of IgE, antileishmanial IgE antibodies as surrogate markers of IL-4 expression were detected only in Brazilian patients with active VL caused by *L. chagasi* and not those with subclinical infection, establishing the diagnostic and prognostic value of specific IgE levels (Atta et al., 1998).

In India, patients with VL have elevated levels of *Leishmania donovani* antigen-specific antibodies including IgG, IgM, IgE and IgG subclasses (Anam et al., 1999a; Ghosh et al., 1995a). Further, in attempting to associate antibody isotypes with progression and cure of disease, a study demonstrated that only 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.
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remained elevated in all patients (Anam et al., 1999b). Another Indian study focusing on *Leishmania*-specific IgG and IgG subclasses in antimony resistant and responsive cases found increased levels of total IgG and four subclasses in the order IgG1 > IgG2 > IgG3 = IgG4 (Chatterjee et al., 1998). Importantly, significantly reduced antibody levels were reported in antimony-responsive cases with no change or a modest increase in resistant cases; profiling of IgG1 and IgG2 levels in Indian VL patients was suggested as a serological approach for monitoring disease status (Chatterjee et al., 1998).

Regional variations in isotype and subclass profiles among VL patients are evident from an Ethiopian study that pointed to raised levels of IgG3 and IgG4 and to a lesser extent, IgG1 antileishmanial antibodies as immune markers for patients with active disease as opposed to subclinically infected and cured individuals (Hailu et al., 2001). To buttress the point, staying within the same region, Sudanese studies on IgG subclass distribution in VL patients prior to and after treatment with pentavalent antimony proposed IgG1 and IgG3 levels as serological markers of cure (Elassad et al., 1994).

It is questionable whether these elevated levels of *Leishmania*-specific antibodies contribute in any way to protection against VL. Miles et al. (2005) sought to answer this question with a study on IgG deficient BALB/c mice by demonstrating resistance to infection with *L. major* as compared to normal BALB/c mice. They were thus able to show that instead of offering protection of any sort, anti-Leishmania IgG actually drove disease progression by virtue of its capacity to stimulate IL-10 production in macrophages.

Although a strong humoral response appears to be of no benefit to the host, quantifying the IgG subclass levels may serve to pinpoint the role(s) of different cytokines in pathology, as differentiation, maturation and proliferation of B lymphocytes is directed by T cells and their secreted messenger molecules, the cytokines. The surrogate kinship between the antibody subclasses and immunological responses has been documented in cutaneous leishmaniasis (Rodriguez et al., 1996) and in antimony-resistant visceral leishmaniasis where the unaltered post-treatment levels of IgG1, IgG2, IgG3 and IgG4 levels was proposed to reflect the increased levels of IFN-γ, IL-4 and IL-10 (Chatterjee et al., 1998).
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The humoral picture in PKDL?

Diagnosis of PKDL can often prove difficult as the definitive parasitological yardstick of demonstration of Leishman-Donovan (LD) bodies in skin lesions does not yield satisfactory results as parasites are not detectable, especially in the hypopigmented macular lesions. In this context, serological diagnostic assays tapping the residual humoral response of PKDL patients have come into prominence with a view to smoothen the diagnostic issues of PKDL vis-à-vis leprosy among other diseases.

In one of the earliest serological studies on Indian PKDL, serum IgG and IgM were significantly increased as compared to healthy controls while they were much lower vis-à-vis VL patients (Haldar et al., 1981). Subsequently, while Ghosh et al. (1995a) showed increased antileishmanial IgG1, IgG2 and IgG3 (in that order) levels with minimal IgG4 to be characteristic of both VL and PKDL sera in India, Saha et al. (2005) documented raised anti-*L. donovani* IgG, IgM, IgA, IgG1, IgG2, and IgG3 antibodies in PKDL sera as compared to cured VL sera; further, patients with PKDL showed no antileishmanial IgE and IgG4, unlike patients with active VL.

As PKDL follows VL in a small percentage of cases after treatment, it would be interesting to look at the surrogate relationship between the IgG subclasses and the cytokines in the PKDL milieu and seek out newer immune markers that may be altered in the transition from VL to PKDL.

Study objectives

We have profiled the humoral response of patients with PKDL that included their immunoglobulin distribution (total IgG and subclasses) and compared with VL patients and healthy controls. This was aimed at establishing whether there was any correlation between the IgG subclasses and development of macular/papular/nodular lesions. Another objective was to study observed differences, if any, in the IgG subclasses between patients with PKDL and VL, before and after treatment, as compared to healthy individuals. Since IgG subclasses function as surrogate markers for pro- and anti-inflammatory cytokines, their profile in PKDL patient sera could potentially serve as a pointer to the interplay of those counteracting cytokines relevant to the immunopathogenesis of PKDL.
Materials and Methods

Collection of serum

After separation, sera were stored in aliquots at -20°C until use.

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.

Indirect ELISA

Total anti-leishmanial IgG binding to LDA was detected using horseradish peroxidase (HRP) conjugated Protein A, as described in Materials and Methods.

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.

Cytokine ELISA

Levels of IFN-γ and IL-10 in sera were quantified by sandwich ELISA using anti-cytokine monoclonal antibodies and binding detected by streptavidin-HRP, as detailed in Materials and Methods.
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Results

Anti leishmanial IgG raised in PKDL but lower compared to VL

Sera from patients with PKDL (n = 16), VL (n = 7) and healthy controls (n = 10) were assayed for total anti leishmanial IgG. Antibody levels in terms of OD$_{405}$ (Figure 4.1) in patients with PKDL although significantly higher compared to healthy controls (1.06 ± 0.64 vs. 0.08 ± 0.02, p < 0.01) were significantly lower than levels in patients with VL (1.92 ± 0.62, p < 0.01).

Figure 4.1. Total IgG levels in patients with PKDL and VL.

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Total anti-leishmanial IgG in patients with PKDL (n = 16), VL (n = 7) and healthy controls (n = 10) was determined by ELISA as described in Materials and Methods. Columns represent mean values; error bars denote SEM. **p ≤ 0.001, *p < 0.01, significantly different from controls; @p < 0.01, significantly different from VL.
IgG subclass profiles: Anti leishmanial IgG3 levels peak in PKDL

Sera from patients with PKDL (polymorphic, n = 11; macular, n = 5), VL pre-treatment (n = 7), VL post-treatment (n = 6) and healthy controls (n = 10) were analysed for their anti leishmanial IgG subclass distribution. In all patient groups, barring macular PKDL and post-treatment VL, levels of IgG1 were significantly higher than controls (0.06 ± 0.02, Figure 4.2), VL patients demonstrating the highest increase (1.52 ± 0.29, Figure 4.2). Interestingly, IgG1 levels in polymorphic PKDL (0.51 ± 0.11) were significantly lower compared to VL patients (p < 0.01) and were comparable to levels in macular PKDL (0.15 ± 0.07) or cured VL (0.36 ± 0.09).

The IgG2 levels were minimally increased in polymorphic and macular PKDL, absorbances being 0.11 ± 0.03 and 0.11 ± 0.04; the VL group showed a slight increase (0.16 ± 0.06) as compared to controls (0.06 ± 0.02) which regressed upon cure (0.07 ± 0.035; Figure 4.2). With regard to IgG3 levels, they were significantly higher in patients with PKDL and VL (pre- and post-treatment) as compared to controls (0.18 ± 0.03, p < 0.0001), highest levels being in polymorphic PKDL (1.64 ± 0.14) followed by macular PKDL (1.25 ± 0.21). VL patients (1.19 ± 0.15) showed a significant decrease in IgG3 levels following treatment (0.69 ± 0.08, p = 0.02; Figure 4.2). IgG4 was minimal in PKDL patients but in VL patients, IgG4 levels were significantly raised (0.45 ± 0.13) as compared to controls (0.05 ± 0.012, p = 0.002; Figure 4.2). Taken together, our data indicate that in Indian PKDL, the subclass order is IgG3>>IgG1>>IgG2>>IgG4 whereas in VL, the subclass order is IgG1 > IgG3>>IgG4>IgG2.
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Figure 4.2. IgG subclass profiles in patients with PKDL and VL before and after treatment.

Distribution of anti-leishmanial IgG1, IgG2, IgG3 and IgG4 in patients with polymorphic (■, n = 11) or macular (▲, n = 5) PKDL, VL (▼, n = 7), post-treatment VL (●, n = 6) and healthy controls (♦, n = 10) was determined by ELISA as described in Materials and Methods. Horizontal lines indicate mean values.

* p < 0.01, ** p < 0.001, *** p < 0.0001, significantly different from controls; 
® p < 0.01, significantly different from VL; “p < 0.05, “p < 0.001, significantly different from Post VL.
Humoral response in PKDL to treatment with Miltefosine or sodium antimony gluconate (SAG): Total anti-leishmanial IgG, IgG1 and IgG3 levels regress upon treatment.

Sera from the 2 groups of patients were tested for anti-leishmanial IgG (total) and its subclasses. Patients who received Miltefosine had significantly reduced IgG levels following treatment (0.68 ± 0.07 as compared to 0.99 ± 0.08 at presentation, p = 0.0003, Figure 4.3), as did patients treated with SAG (0.44 ± 0.11 vs. 0.87 ± 0.19 at presentation, p = 0.056, Figure 4.3).

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, IgG1 levels, like total IgG, showed significant reduction after treatment with Miltefosine (1.16 ± 0.26 at presentation vs. 0.89 ± 0.23, p = 0.006), and a similar trend was obtained with SAG although differences were not statistically significant (0.69 ± 0.21 at presentation vs. 0.26 ± 0.1, p = 0.14). Treatment with Miltefosine reduced IgG3 levels significantly from 0.81 ± 0.19 at presentation to 0.35 ± 0.13 (p = 0.039), as did treatment with SAG from 1.86 ± 0.22 before treatment to 1.09 ± 0.22 (p = 0.033). IgG2 and IgG4 levels, as shown earlier, were barely detectable in patients at presentation and remained so after treatment (Figure 4.3).
Figure 4.3. Total IgG and subclass profiles in patients with PKDL before and after treatment.
Serum cytokine profiles: IFN-γ and IL-10 are elevated in polymorphic PKDL.

To quantify serum levels of counter-regulatory cytokines in PKDL patients, sandwich ELISAs for IFN-γ and IL-10 were performed. In polymorphic PKDL, IFN-γ levels (pg/ml) were significantly higher compared to controls (128.70 ± 18.69 vs. 53.19 ± 1.16, p = 0.002) and macular PKDL (56.27 ± 2.32, p = 0.02) but were comparable to VL patients (92.73 ± 17.39). VL patients, upon cure, continued to have significantly elevated IFN-γ levels (108.9 ± 3.9) as compared to controls (p < 0.0001) and macular PKDL (p < 0.0001, Figure 4.4). IL-10 levels were significantly raised in both forms of PKDL (146.70 ± 21.67 in polymorphic and 61.75 ± 2.07 in macular PKDL, p < 0.005) and VL patients, before and after cure (103.60 ± 19.30, p < 0.05 and 104.00 ± 3.98, p < 0.0001, respectively), as compared to controls (51.83 ± 1.76); notably, levels of IL-10 in macular PKDL were again significantly lower than polymorphic PKDL (p < 0.05) and VL post-treatment groups (p < 0.0001, Figure 4.4).
Serum cytokine profiles in PKDL patients remain unaltered following treatment

To determine the effect of treatment with Miltefosine or SAG upon serum cytokine levels, sandwich ELISA was performed. It was observed that the elevated cytokine levels seen at disease presentation were not affected by treatment, irrespective of the drug received by the patients (Figure 4.5). However, there was a moderate but insignificant decrease in extracellular IFN-γ levels with Miltefosine (from 334.9 ± 17.35 to 299.9 ± 5.451, p = 0.09), together with a modest reduction in IL-10 levels (from 229.7 ± 3.66 to 222.1 ± 4.82). Further, there was no correlation between serum IL-10 levels and antileishmanial IgG3 levels in patients with PKDL before and after treatment (data not shown).

Figure 4.5. Serum cytokine levels in patients with PKDL before and after treatment.
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Discussion

Previous studies have detected the IgG class as the predominant class of antibodies in sera of Indian VL and PKDL patients (Ghose et al., 1980; Haldar et al., 1981). Lower total anti-Leishmania IgG in patients with PKDL as compared to VL has been documented by Haldar et al. (1981). We corroborated this trend in our study, showing significantly raised IgG in PKDL compared to healthy controls, which was at the same time significantly lower compared to VL (Figure 4.1). Ideally, the most appropriate control group for patients with PKDL would be cured VL patients who have not developed PKDL. However, our limitation is that Indian PKDL appears after a long and unpredictable intervening period making it nearly impossible to follow up VL patients. For instance, one of the patients in this study had VL 11.5 years back and understandably, tracking an apparently healthy individual cured of VL 11.5 yrs ago would not be feasible. Investigations entailing extensive follow-up of VL patients 1-2 years after cure would definitely yield interesting data and should be included in future studies on Indian PKDL. In the present study, for uniformity, VL post-treatment samples were collected 1 month after completion of treatment. Although higher than PKDL, antileishmanial antibody levels in VL, a systemic disease, often vary, possibly due to differences in duration and clinical severity (Ghosh et al., 1995a). It has been proposed that the limited serological response of PKDL patients is due to the localized nature of the disease (Haldar et al., 1981), as is evident in our findings.

Cytokines play a crucial role in the maturation of B lymphocytes into antibody secreting plasma cells. IL-4 helps mediate IgG4 and IgE synthesis (Gascan et al., 1991) while IL-10 is associated with enhanced IgG1 and IgG3 levels (Briere et al., 1994). On the other hand, IFN-γ together with IL-6 causes an increase in IgG2 levels (Kawano et al., 1994). Thus, IgG isotypes can double as surrogate serological markers for various cytokines. In a recent study in PKDL patients, the order was IgG1 > IgG3 > IgG2 vis-à-vis healthy, endemic and other disease controls (Saha et al., 2005). However, we show that both sub-groups, polymorphic and macular PKDL, consistently exhibited raised IgG3 levels whereas IgG1 levels dominated in the VL group and we report the order as IgG3 > IgG1 > IgG2 in case of PKDL and IgG1 > IgG3 > IgG2 in case of VL (Figure 4.2). Although IgG1 levels were comparable in patients with PKDL and post-VL patients, IgG3 levels in the latter were lower, compared to both polymorphic and macular PKDL. This suggests an etiologic association for this particular IgG subclass.

The findings are of particular interest as the principal anti-inflammatory cytokine, IL-10, is known to induce secretion of IgG1 and IgG3 by naïve B cells in humans (Briere et al., 1994; Fujieda et al., 1996). Furthermore, IgG1 and IgG3 production in humans is also strongly associated with IL-10 secretion in parasitic diseases (Caldas et al., 2005). Why one IL-10 marker is elevated in PKDL (IgG3 > IgG1) and the converse true in VL
(IgG1>IgG3) emphasizes the need for an investigation into the functional significance of the IgG isotypes in VL and PKDL. Taken together, the observed increase in serum IL-10 in PKDL patients was concomitant with enhanced IgG1 and IgG3 levels, which are known to be driven by IL-10 (Briere et al., 1994; Fujieda et al., 1996). Clearly, IL-10 has a significant role in promoting pathogenesis of Indian PKDL.

Counteracting pro- (IFN-γ and IL-12) and anti-inflammatory (IL-10) cytokines in circulation have been reported in VL cases worldwide (Sundar et al., 1997; Caldas et al., 2005). Ansari et al. (2006a) have reported that in PKDL, serum levels of IFN-γ, IL-10 and IL-6 as measured by cytometric bead array were lower than in VL patients and similar to healthy controls, suggesting that the immune response in PKDL is localized. However, our data differs in that differences do exist systemically in Indian PKDL as IFN-γ and IL-10 levels were higher in PKDL patients presenting with polymorphic lesions as compared to both controls and patients with macular lesions (Figure 4.4). As patients with PKDL have had a past history of VL, their serum cytokine profiles are best compared to post-treatment VL patients. Accordingly, serum IFN-γ and IL-10 levels in post-treatment VL patients were significantly higher than macular PKDL but comparable to polymorphic PKDL. This probably reflects the difference between the two clinical forms of PKDL both in disease severity (parasite persistence being favoured by IL-10) and associated inflammation (elevated IFN-γ as part of the host’s response against thriving parasites). Within the polymorphic group, there was a small subgroup of patients who had markedly higher levels of both IFN-γ and IL-10 and as such, appear to contribute significantly to the overall increase in group serum cytokine levels. Considering the small size and the observed immunological heterogeneity of the patient population, their immunological profiles would best be established by assessing a sizeable number of patients with PKDL over a period of time, given the low frequency of the disease. To date, there have been no reports on peripheral cytokine level distinctions in the two distinct clinical forms of PKDL and this is the first such report on PKDL, irrespective of origin.

We assessed the performance of two drugs, the treatment mainstay, sodium antimony gluconate (SAG) faced with increasing problems of resistance and toxicity, and the newer miltefosine, on the basis of observed improvement in several immunological parameters two of which were serum IgG with its subclasses and serum cytokines. With respect to the humoral response to treatment, miltefosine was successful in lowering total IgG, IgG1 and IgG3 levels, the fold reduction being more significant statistically as compared to SAG (Figure 4.3). Additionally, miltefosine had a small but insignificant lowering effect on serum IFN-γ and IL-10 (Figure 4.5). Taken together, miltefosine was equally if not more effective than SAG in lowering several important immunodeterminants of PKDL.