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Whole blood Assay and Visceral Leishmaniasis: Challenges and Promises

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Abstract

For years, the ability to study immune responses in patients with active visceral leishmaniasis (VL) has been hampered by the absence of detectable antigen-specific Th1 responses using cells from peripheral blood mononuclear cells (PBMCs). Employing whole blood assay (WBA), we recently reported that whole blood cells of active VL patients maintain the capacity to secrete significant levels of antigen driven IFN- γ and IL-10. Furthermore, WBA that uses soluble leishmania antigen (SLA) have advantages over the leishmanin skin test (LST), in terms of higher specificity and better correlation with surrogate markers of exposures to *L. donovani*. These findings open the door to a series of immunological and epidemiological studies not previously possible for VL. In the present review, we discuss current status, future perspectives as well as obstacles in the research on WBA. Research in this area is essential for development of potential immunological and epidemiological tools for VL.

Keywords

Whole blood assay; Visceral leishmaniasis; SLA; IGRA; LST

Introduction

Visceral leishmaniasis (VL) or kala-azar is a parasitic infectious disease that is fatal if left untreated. The estimated annual global incidence of VL is 200,000 to 400,000 and >90 % of these cases occur in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Alvar *et al.* 2012). Interestingly, 80 to 90% of human infections are subclinical or asymptomatic and this asymptomatic infection is characterized by a positive leishmanin skin test in studies emanating from Brazil, Ethiopia and Sudan (Badaro *et al.* 1986; Hailu *et al.* 2009; Jeronimo *et al.* 1994; Jeronimo *et al.* 2000; Satti *et al.* 2002). Investigation of cellular immunity and T cell function in humans is usually restricted to studies on peripheral blood mononuclear cells (PBMCs). T-cell-specific immune responses are primarily induced by the interaction of processed antigen with the T-cell receptor (TCR) / CD3 complex; and separation of T cells from a physiological environment is likely to have intense modifying effects on T-cell function and can even cause pre-activation (Petrovsky & Harrison 1995). Whole-blood assays (WBA) may overcome these limitations, since they contain all cell populations and soluble factors needed for T cell activation and therefore most closely mimic *in vivo* conditions. Importance of T cells in the protective immune response against *Mycobacterium tuberculosis* (Mtb) has long been known since their major function is the production of interferon- γ (IFN- γ) and tumor necrosis factor (TNF- α), which have been verified to play a

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crucial role in macrophage activation, control of mycobacteria replication and granuloma formation, both in humans and mice (Stenger & Modlin 1999). Protective immunity to *Leishmania* infection is predominantly T cell-mediated and results in the killing of intracellular parasites by activated macrophages and cytotoxic responses (Peruhype-Magalhaes *et al.* 2006). Several groups have developed WBA for measuring responses to mitogens (De.Groote *et al.* 1992; Junge *et al.* 1970; Petrovsky & Harrison 1995), antigens (Paty & Hughes 1972; Pauly *et al.* 1973) and specific antigens for investigating a variety of infectious diseases in which T cell mediated immunity plays an important role including herpes virus (Leroux *et al.* 1985), various bacterial infections (Kaneene *et al.* 1978; Koskela & Herva 1980), leprosy (Weir *et al.* 1998; Weir *et al.* 1994; Weir *et al.* 1999) and tuberculosis (Diel *et al.* 2011; Fiavey & Frankenburg 1992). However, we have recently developed and optimized the whole blood interferon- γ release assay (IGRA) for VL, using soluble leishmania antigen (SLA); which permits the measurement of cytokines released from stimulated monocytes & T-cells (Ansari *et al.* 2012; Gidwani *et al.* 2011; Singh *et al.* 2012a). The test is based on the principle that T-cells from the whole blood sample, when exposed and incubated with a leishmania specific antigen produce IFN- γ (Box-1). The test is performed by obtaining heparinized whole blood and incubating it with leishmania peptide (e.g. SLA). Other antigens, such as phytohemagglutinin (PHA) and saline (PBS) are used as controls. The former is a mitogen that, after stimulating whole blood serves as an IFN- γ positive control for each specimen; the latter is a control that adjusts for heterophile antibodies in serum or plasma, which are known to cause interference with immunoassays (Mazurek *et al.* 2010). After 16 to 24 hours, production of IFN- γ in culture supernatant is measured by ELISA. The test is reported as positive, negative or indeterminate by the cut-off value determined by ROC curve (Singh *et al.* 2012a). Indeterminate results can be caused by reduced lymphocyte count in the blood sample or reduced lymphocyte activity because of an intercurrent illness (such as human immune-deficiency virus [HIV] infection, malignancy, or immunosuppressive drugs); prolonged specimen transport or improper specimen handling; and incorrect addition of the mitogen. Such a rapid and simple test would be especially useful for VL studies, since VL is largely a disease confined to developing countries where laboratory facilities are often limited. In the present review, promises and challenges of this new assay are discussed with particular emphasis on immuno-epidemiological perspectives of WBA based cytokines profiling for visceral leishmaniasis.

Immunological basis of IGRA

IGRAs exploit key immunological mechanisms that occur upon infection with *Leishmania* antigen and allow detection of the host adaptive immune response to the pathogen *ex vivo*. During natural *Leishmania* infection, antigens are presented to T-cells via major histocompatibility complex (MHC) molecules on the APC surface. Presentation of *Leishmania* specific antigens via MHC class II molecules to CD4+ T cells activates cells expressing a T-cell receptor (TCR) specific to the MHC - *Leishmania* antigen complex, and leads to differentiation of *Leishmania* specific CD4+ T cells into effector and memory T helper cells. Differentiated effector T cells migrate to infected tissues, where they drive a T helper1 (Th1) biased inflammatory response; memory T cells reside in the lymph nodes, maintaining long-term immunological memory of *Leishmania* infection. *Leishmania* antigen presentation by APCs to CD8+ T cells via MHC Class I molecules leads to differentiation of cells expressing a TCR specific to the MHC-*Leishmania* antigen complex into cytotoxic T cells.

Whole blood Assay and Immunopathology of human VL

Visceral leishmaniasis is associated with a marked depression of T-cell responses, which has been characterized by the absence of IL-2 and IFN- γ production by PBMCs on *in vitro*

stimulation with *Leishmania* antigen and is thought to underlie the progressive nature of this disease (Bacellar *et al.* 1996; Caldas *et al.* 2005; Carvalho *et al.* 1985; Halder *et al.* 1983; Ho *et al.* 1992; Sacks *et al.* 1987; Saha *et al.* 2007). The conclusion that this immune unresponsiveness reflects the immunologic deficit that underlies disease progression in the VL patients is widely accepted. However, there appears to be no inherent defect in antigen-induced Th1 responsiveness because cured individuals are resistant to re-infection, become leishmanin skin-test positive and mount antigen-specific IFN- γ responses *in vitro* (Nylen *et al.* 2007). It should be noted that antigen driven Th2 or IL-10 responses have been equally difficult to detect in cultures of PBMCs from VL patients. In contrast, PBMCs from the majority of cured patients proliferate and/or produce IFN- γ or TNF- α in response to antigen (Caldas *et al.* 2005; Ghalib *et al.* 1993; Ho *et al.* 1992; Sacks *et al.* 1987; Saha *et al.* 2007).

The results of a series of our recent studies, in which significant secretion of both IFN- γ and IL-10 was detected in antigen stimulated cultures of peripheral whole blood cells from active VL cases, opens the door to a series of immunologic studies not previously possible, including a comprehensive analysis of antigen driven signature genes by transcriptional profiling or multiplex analysis of secreted cytokines and chemokines in patients with different disease severity and following clinical cure. These findings also suggest that unfavorable clinical outcomes are not related to an intrinsic defect in Th1 response *per se*, but that other immunosuppressive or immune evasion mechanisms contribute to the pathogenesis of VL (Ansari *et al.* 2012; Gidwani *et al.* 2011; Singh *et al.* 2012a). However, concomitant production of Ag-specific IFN- γ and IL-10 suggests that, although essential for acquired resistance to *L. donovani*, IFN- γ might also be involved in the regulation of T cell IL-10 expression as a homeostatic mechanism to restrain inflammation.

Clinically, patients with active disease have elevated levels of IL-10 in serum as well as enhanced IL-10 mRNA expression in target organs such as the spleen or bone marrow. The WBA findings further support the view that IL-10 is key immunosuppressive cytokine in VL patients, since only patients with active disease responding in WBA by secreting increased levels of IL-10 upon stimulation with SLA. WBA provides the direct evidence that the IL-10 is produced by antigen specific cells, and is consistent with our previous finding that CD4⁺CD25⁺Foxp3⁺ T cells were the main source of elevated IL-10 mRNA present in the VL spleen (Nylen *et al.* 2007). Furthermore, employing the WBA, we were also able to demonstrate a modest but consistent enhancement of IFN- γ secretion by antigen stimulated whole blood cells from VL patients in the presence of neutralizing anti IL-10 antibodies (Singh *et al.* 2012a). Our inability to observe such effects in prior studies employing PBMCs is perhaps not surprising given that we failed to detect IL-10 secretion by the antigen-stimulated cells. Taken together, these findings strongly suggest that the elevated expression of IL-10 might inhibit Th1 cell activation in human VL, either directly or via effects on APC function. Despite the relatively high levels of IFN- γ secretion by SLA stimulated whole blood cells from active VL cases, the fact that IL-10 was simultaneously secreted, suggests that the IFN- γ response might still have been compromised. Nonetheless, based on the comparable levels of IFN- γ produced by active and cured VL patients that co-express or not IL-10, we would argue that the main disease promoting activity of IL-10 in VL might be to render host macrophages refractory to activation signals in response to IFN- γ and/or TNF- α , both of which are produced by antigen stimulated whole blood cells from VL patients. Strong evidence that IL-10 prevents the efficient clearance of *L. donovani* in human VL was recently provided in studies showing that the parasite numbers present in splenic aspirate cells were markedly reduced following a short term culture in the presence of anti IL-10 monoclonal antibodies (Gautam *et al.* 2011).

T cell source of antigen-stimulated IFN- γ produced by the whole blood cells in these assays has been confirmed (unpublished data). However, the difference between the T cell

responsiveness in whole blood as opposed to PBMC preparations is not yet understood. Interestingly, co-cultures of PBMCs obtained prior to the patient's treatment suppress the lymphocyte proliferation of cells to *Leishmania* antigen obtained from the same patient after successful cure, suggesting there are immunosuppressive factors produced by these PBMCs (Carvalho *et al.* 1989). Studies from South America and Sudan, antigen specific unresponsiveness in PBMCs from VL patients with respect to T-cell proliferation and IFN- γ production has been observed to be reversed by treatment with anti-IL-10 antibodies (Carvalho *et al.* 1994; Ghalib *et al.* 1993; Ghalib *et al.* 1995). It is not clear from these studies, however, which cells were proliferating or the source of IFN- γ . In our previous studies of a large series of VL patients from India, we were unable to detect antigen driven IL-10 production by PBMCs or to recover an antigen-specific response with anti-IL-10 treatment (Nylen *et al.* 2007). Further studies are clearly needed to elucidate whether antigen-specific T cells are present but suppressed in active VL, if they are lost and/or never generated appropriately or if they are recruited to the sites of infection and are therefore not detectable in the PBMCs.

Clinical and epidemiological aspects

Achievement of the World Health Organization (WHO) goal for global elimination of leishmaniasis as a public health problem is hindered by the lack of information on the presence of *Leishmania donovani* infection within a given population (WHO-2005). Control programmes are guided by treatment of cases of VL, and surveillance of their close contacts who have been shown to suffer from a higher incidence of VL than the general population, but by no means account for all new cases of VL. It has been suggested that in an area of high VL endemicity there may be no difference in incidence between contacts and the general population (Bart *et al.* 2011). Currently, it is difficult to predict exactly who among the subclinically infected people will develop the disease and when (Gidwani *et al.* 2009a). Therefore, a specific test for *Leishmania* infection is needed to answer some of these fundamental questions in VL epidemiology, and helps to VL control programmes, including targeted vaccination programmes with current or future vaccines. Approaches to date, including serology may have not been able to achieve sufficient specificity and sensitivity while being robust and simple enough for use in rural VL endemic areas (Chappuis *et al.* 2007). Although lymphocyte proliferation is a well-known technique to measure cell-mediated immunity *in vitro*, it has only been used to a limited extent in the study of infectious diseases. This is mainly due to the relatively large amounts of blood that are required (5-10 ml) and the relative complexity of the test, due to the necessity to separate the mononuclear cells from the total blood prior to cell incubation. These two considerations hamper the use of lymphocyte proliferation as a routinely performed assay for the diagnosis, follow-up and epidemiological studies of VL in which cell-mediated responses play a role. Leishmanin Skin Test (LST) is a relatively straightforward measure of cell-mediated delayed-type reactivity, however, the current scarcity of GMP-grade leishmanin antigen and its low sensitivity when evaluated on Indian patients are major drawbacks (Gidwani *et al.* 2009b). Employing the whole blood, for the first time we reported that interferon- γ release assay using SLA could potentially replace the LST as a marker of cellular immunity to detect infected and non infected persons with high accuracy (Table-1) (Gidwani *et al.* 2011; Singh *et al.* 2012a) and comparable as in other infectious diseases (Table-2). The findings were also further supported by antibody response against salivary gland antigens of the vector *P. argentipes*. IGRA positive endemic healthy individuals (EHC) had significantly elevated serological titer of antibodies against *P. argentipes* saliva compared to the IGRA negative EHCs or the NEHCs, and comparable to the active VL cases, presumably reflecting their greater exposure to sand fly bites and their greater risk of having been exposed to an infected sand fly (Singh *et al.* 2012a; Clements *et al.* 2010). Furthermore, in recent studies, we have also highlighted the possibility of such whole blood based IGRA on microtiter plate

with less amount of blood (Singh *et al.* 2012b), providing the means to more efficient screening in large-scale epidemiological studies as has been used previously in studies of mycobacterial diseases (Black *et al.* 2001a; Black *et al.* 2001b; Black *et al.* 2002). The whole blood assay, if further validated in larger community based studies, could become a useful immuno- epidemiological tool to provide a more complete picture of *L. donovani* transmission and may contribute to the development of better markers for infection to understand T cell immune response in the high endemic foci of VL.

Technical aspects and future directions

In theory, interferon- γ assay have several advantages over LST. In practice, it is not easy to show the superiority of IGRA over LST. In the absence of gold standard to define *L. donovani* infection in asymptomatic people seriously hampers the development of and the evaluation of the accuracy of new markers. Our reports with IGRAs, however, make clear that antigen driven IFN- γ release is not on its own an adequate marker for immune status, since all active VL and clinically exposed groups produced comparable levels of this cytokine. Other cytokines and chemokines (including IL-2, IL-10, TNF- α , IL-13, IL-5, IP-10, IL-8, IL-21, IL-17, and IL-35) have shown promise as diagnostic markers, but the potential added benefit of these has not yet been systematically assessed (Anderson *et al.* 2009; Fröhlich *et al.* 2007; Gupta *et al.* 2011; Kedzierski *et al.* 2008; Nateghi Rostami *et al.* 2010; Vitale *et al.* 1992; Wilhelm *et al.* 2005). Therefore, by extension to multiple platforms that screen multiple cytokines and chemokines would provide an even more accurate picture of the immune responses that correlate with active disease or clinical cure. IGRA utilizes phytohemagglutinin (PHA) as mitogen positive control. However, PHA may not be the optimal choice for another cytokines test. For example; PHA is a powerful inducer of IFN- γ , whereas interferon inducible protein-10 (IP-10) is induced at relatively lower levels. The difference is probably because PHA acts directly on the T cells but not on the APCs. Therefore, more suitable positive control mitogen needs to be optimized for multiple cytokines platform. Tweaking of the incubation step, for example by increasing incubation temperature (Aabye *et al.* 2011), prolonging the incubation period (Butera *et al.* 2009; Leyten *et al.* 2006), improving culture conditions with nutrients (Rothel *et al.* 2004) or blocking of anti-inflammatory cytokines e.g. IL-10 (Bhattacharjee *et al.* 2009; Gautam *et al.* 2011; Murray *et al.* 2005; Singh *et al.* 2012a), have also shown potential for augmentation of the antigen-specific immune response.

A major limitation in this field relates to the technical aspects of cut-off determination for the assays used for IFN- γ or IL-10 detection. IFN- γ and IL-10 has been measured with a variety of assays: from different brands of multiplexing assays to commercial or in-house ELISA kits. Cutoffs vary from study to study, and as cutoffs are linked both to the readout platform and the sample dilution, a more stringent approach is needed before studies can be compared. Future studies should measure and report cytokines results in ways that allow comparison between studies. Not surprisingly, the best cutoff generated in our studies (by cured VL & NEHC) was different from that described by Turgay *et. al.*, who firstly described the application of IGRA technology in *Leishmania* (Turgay N *et al.* 2010). They used a pool of peptides covering the amino acid sequence of histone H2B, and found 83 % sensitivity with respect to cured VL. Recent study from Iran has shown that the LST is significantly more sensitive than IFN- γ levels in persons who have been cured of cutaneous leishmaniasis (CL) (Alimohammadian *et al.* 2012). However, our previous work showed 43% sensitivity with H2B antigen in India (Gidwani *et al.* 2011) and we therefore preferred to develop the assay with SLA. The cutoff value is likely to be unique for each antigen, and even with each preparation of antigen involving such heterogeneous material as SLA. Efforts should be made to improve the performance of such assay by making specific

leishmania recombinant proteins, as the recombinant antigens have considerably improved sensitivity and specificity over crude/total antigens.

Another controversial topic is T-cell response kinetics during and after treatment for active VL. Recently, we have established and validated whole blood as a suitable source of infected tissue to quantify the infection burden in VL patients before and after treatment (Sudarshan *et al.* 2011). Therefore, combination of whole blood based cytokine production with infection burden or degree of leishmania infection would further be used to identify the immune parameters that correlate with disease severity. Such assays might provide a more quantitative and dynamic assessment for measuring immune responses to leishmania antigens in VL and this potential may be exploited to study the effect of new vaccine and therapeutic agents.

Current evidence suggest IGRA have higher sensitivity and specificity than LST but one of the greatest advantages of the leishmanin skin test is that assessment of T cell mediated immunity has been established in many cohort studies for various populations and associated clinical conditions. Currently, there are no equivalent data for interferon- γ release assay. Thus, a crucial unresolved issue is whether IGRA have the ability to identify cell immune individuals and likely to direct *Leishmania* vaccine research. Recently, Schnorr *et al.* reported that in addition to LST, the sensitivity to determine exposure to *Leishmania* infection can be increased with IGRA in CL (Schnorr *et al.* 2012). Therefore, more studies in other countries are needed to address this essential knowledge gap and to explore the possibility of using both LST and IGRA in combination.

There are virtually no data on the short-term and long-term reproducibility of IGRA, particularly within subjects variability in serial testing, where conversion and reversion can occur. Studied need to distinguish between biological variability of positive responses (i.e. whether they often fluctuate above and below the limit of detection or cut-off for positivity), and frequency of reversions and false positive results as a result of such fluctuations. Thus, long- term cohort studies are needed to better define the role of IGRA in serial testing. Table-3 lists the specific questions relevant to IGRA future studies.

Conclusions

Development of an immuno-epidemiological tool to detect *L.donovani* infection would greatly benefit VL control programmes, as demonstrated by the contribution of the IGRA test in tuberculosis control. WBA provide a simple tool for assessing immune cytokine profiles, which may be useful laboratory predictors of early infection, helping the evaluation of new leishmania vaccines and offering insights into the disease pathogenesis. Several approaches need to be developed to overcome the shortcomings of the current IGRAs or WBA. Alternative or additional antigens may have the potential for improving IGRA sensitivity and enable discrimination between active and latent VL. However, more studies with a broader consensus on several technical aspects in WBA methodology including well-designed trials, long-term follow-up studies and using validated cutoffs, as well as studies in exposed individuals are needed before conclusions can be drawn. Because interferon- γ assays might cost much more than the serological tests, it will be a critical factor in determining the global applicability of this new assay. It will then be important to ensure that the benefits of this new technology, if shown to be valuable, reach the populations that need it most. There is a need to simplify interferon- γ release assay technology or develop alternative platform that will enhance applicability in resource limited and field settings.

New technologies are emerging, and a new generation of rapid and user-friendly tests appears eminent. If proven reliable for large-scale use, these new tests will probably be combined with a novel generation of *Leishmania* specific antigens with higher sensitivity,

and antigens that are able to discriminate between patients with active VL and latent VL infection. In addition, several other uses of WBA could also be studied more thoroughly, such as the potential of the acute-phase reactant properties of plasma cytokines in monitoring treatment responses, and predicting disease severity, mortality and morbidity.

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Box 1**Essential components in the whole blood based IFN- γ release assay test**

- ❖ **Antigens:** the sensitivity and specificity of IFN- γ release assay is primarily linked to the antigens (Gidwani *et al.* 2011).
- ❖ **Antigen presenting cells (APCs):** APC express MHC molecules with specificity for antigens to T cells. If the T-cell receptor recognizes the peptide antigen on the MHC, both the T-cell and APC become activated.
- ❖ **T cells:** a high number of memory T cells specific for the peptides presented on APCs ensure a strong immune activation *in vitro*.
- ❖ **Incubation and read out biomarker:** for immune activation to occur, the cells need time and heat. The commercial tests incubate for 16-24 hrs. at 37°C and monitor the degree of immune activation by measuring the release of IFN- γ . If the value of IFN- γ is higher than the cutoff (determined by non exposed individuals i.e. NEHC), the test deemed positive; and if there is little or no response, the test is deemed negative (Singh *et al.* 2012a).

Table 1

Comparison of modified QuantiFERON assay (IGRA) and Leishmanin Skin test (LST)

Performance and operational characteristics	Modified Quantiferon assay (IGRA)	Leishmanin Skin test (LST)
How is test performed?	Laboratory measurement of antigen specific IFN- γ in whole blood	Intradermal injection of GMP grade purified protein derivatives and measurement of resulting induration
What does the test measure?	<i>In-vitro</i> release of IFN- γ after exposure to leishmania antigen	<i>In vivo</i> type of intradermal hypersensitivity after injection of leishmanin antigen
Ability to detect leishmania infection	Yes (with high sensitivity)	Yes (but low sensitivity)
Ability to distinguish active VL and cured VL.	Yes (by measuring IFN- γ and IL-10)	No
Need for patients to return of second visit	No	Yes
Required Laboratory infrastructure	Yes (ELISA related equipments)	No special infrastructure required
Cost	More expensive	Less expensive
Time to obtain results	At least 24 hrs.	At least 48 hrs.

Table 2Sensitivity and specificity of whole blood based IFN- γ release assay (IGRA) in infectious diseases.

S.N	Disease	Sensitivity (95% CI)	Specificity (95% CI)	References
1	Visceral Leishmaniasis	82 -86 %	100%	Gidwani <i>et al.</i> 2011; Singh <i>et al.</i> 2012a; Turgay <i>et al.</i> 2010
2	Tuberculosis	85 – 88 %	94 – 98%	Mori <i>et al</i> 2004; Kobashi <i>et al</i> 2006; Pai <i>et al.</i> 2008
3	Tuberculosis + HIV	66 - 100 %	54 – 80%	Kabeer <i>et al.</i> 2009; Dheda <i>et al.</i> 2005; Cattamanchi et al 2010
4	Leprosy	82 %	100 %	Duthie <i>et al.</i> 2008
5	Congenital toxoplasmosis	94%	98%	Chapey <i>et al</i> 2010

Table 3

A research agenda for future work on IFN- γ release assays.

S. N.	Research questions
1	To what extent does a positive IGRA suggest previous infection (either cleared or still active) versus recent active infection? What type of T-cell responses are detected by IGRAs: effector or memory T-cell responses?
2	What is the biological basis for discordance between LST and IGRA results?
3	After treatment of VL patients, how long does it take for the IGRA test to become positive?
4	Can IGRA technology be simplified? For example, testing with smaller quantities of blood (e.g., fingerstick) and lateral flow or strip formats.
5	What is the amount of test related variability in T-cell responses? For example, variations in IFN- γ due to variability of factors such as operators, laboratories, sample processing intervals, incubation times, antigens (SLA vs recombinant proteins).
6	What is the amount of random, biological variability of IFN- γ and IL-10 responses over time within same individuals, including day to day, week to week variability of IFN- γ and IL-10 levels in the absence of leishmania exposure?
7	Can IGRA be used in population survey to estimate annual risk of leishmania infection?
8	In vaccine trials, can IGRA serve as correlates of protective immunity?
9	What is the risk of cross-reaction of IGRA in case of co-infection with other kinetoplastidae infections?