Immunobiology 219 (2014) 323–328

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Review

Whole blood assay and visceral leishmaniasis: Challenges and promises

Om Prakash Singh, Shyam Sundar*

Infectious Disease Research Laboratory, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, India

ARTICLE INFO

Article history:
Received 3 September 2013
Received in revised form 5 December 2013
Accepted 19 January 2014
Available online 31 January 2014

Keywords:
Whole blood assay
Visceral leishmaniasis
SLA
IGRA
LST

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 *Leishmania 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.

Contents

Introduction

Visceral leishmaniasis (VL) or kala-azar is a vector-borne parasitic infectious disease that is fatal if left untreated. The estimated annual global incidence of VL is 200,000–400,000 and >90% of these cases occur in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Alvar et al. 2012). Interestingly, 80–90% of human infections are subclinical or asymptomatic and this asymptomatic infection is characterized by a positive leishmanin skin test (LST) in studies emanating from Brazil, Ethiopia and Sudan (Badaro et al. 1986; Hailu et al. 2009; Jeronimo et al. 1994, 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 and 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 been known long since their major function is the production of interferon-γ (IFN-γ) and tumor necrosis factor (TNF-α), which have been verified to

* Corresponding author at: Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, UP, India. Tel.: +91 542 2369632.
E-mail address: drshyamsundar@hotmail.com (S. Sundar).

0171-2985/$ – see front matter © 2014 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.imbio.2014.01.005

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 h 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).

play a crucial role in macrophage activation, control of mycobacteria replication and granuloma formation, both in humans and mice (Stenger and Modlin 1999). Protective immunity against *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 and Harrison 1995), antigens (Paty and 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 and Herva 1980), leprosy (Weir et al. 1998, 1994, 1999) and tuberculosis (Diel et al. 2011; Fiavey and 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 16–24 h, production of IFN-γ in culture supernatant is measured by ELISA. The test is reported as positive, negative or indeterminate by the cutoff 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 immuno-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 drive T helper1 (Th1) based 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; Haldar 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, open 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 is not due to a defect in the ability to mount protective 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 to 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