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Antigen-specific secretion of IFNγ and CXCL10 in whole blood assay detects *Mycobacterium leprae* infection but does not discriminate asymptomatic infection from symptomatic leprosy

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ABSTRACT

To advance toward a whole blood assay (WBA)-based test capable of facilitating the diagnosis of paucibacillary (PB) leprosy, we evaluated a prototype in-tube WBA using combinations of *Mycobacterium leprae* antigens. Blood was collected from newly diagnosed untreated PB (n = 38), multibacillary (MB) (n = 30), healthy household contacts (HHC) of MB (n = 27), and endemic controls (n = 61) residing in Goiânia and Fortaleza, Brazil. Blood was incubated with *M. leprae* cell sonicate, recombinant proteins (46f + LID-1; ML0276 + LID-1), or controls (phosphate-buffered saline, phytohemagglutinin, *M. tuberculosis* purified protein derivative). Antigen-specific IFNγ production was observed in 71–84% and 55% of PB and HHC, respectively. Antigen-specific CXCL10 levels were similarly assessed to determine if, unlike IFNγ, CXCL10 could differentiate PB from HHC with repeated exposure/asymptomatic *M. leprae* infection. The CXCL10 levels induced in response to *M. leprae* antigens could not, however, differentiate PB from HHC. Despite these limitations, the WBAs reported here still represent important tools for assessing *M. leprae* infection rates and evaluating the impact of control measures.

1. Introduction

Leprosy is a complex chronic dermatoneurological disease caused by *M. leprae*. The hallmarks of leprosy are the disabilities and physical deformities that can occur as a consequence of the unique tropism of *M. leprae* for Schwann cells and also the infection-induced inflammatory response (Scollard et al., 2006). For operational purposes, the World Health Organization (WHO) proposed a simplified classification system based on the number of leprosy skin lesions in which paucibacillary (PB) leprosy present up to 5 lesions and multibacillary (MB) leprosy patients have more than 5 skin lesions. Leprosy is then treated with multidrug therapy, which for PB leprosy patients consists of 6 doses of rifampicin and dapsone given over 6 months, while for MB leprosy patients, it consists of 12 doses of rifampicin, dapsone, and clofazimine given over a year (WHO, 1982).

Despite the consolidation of diagnostic and treatment guidelines to the MB and PB classifications, leprosy actually presents across a wide spectrum of clinical outcomes. These are characterized by distinct clinical, bacteriologic, immunologic, and pathologic features (Ridley and Jopling, 1966). Patients with the polar tuberculoid (TT) form are PB with low or absent bacterial index (BI), have few granulomatous skin lesions, and have low or absent antibody production but strong *M. leprae*-specific Th1 type cell-mediated immunity (CMI). At the other pole, lepromatous (LL) patients are MB with high BI, have multiple disseminated skin lesions, and have vigorous antibody production but low or absent *M. leprae*-specific CMI (Britton, 1993; Rodrigues and Lockwood, 2011; Scollard et al., 2006). It is much more common to observe presentations between these poles in the form of borderline tuberculoid (BT), borderline borderline (BB), and borderline lepromatous (BL).

Abbreviations: AUC, area under the curve; BI, bacterial index; BB, borderline borderline; BL, borderline lepromatous; BT, borderline tuberculoid; BSA, bovine serum albumin; CMI, cell-mediated immunity; CXCL10, C-X-C motif chemokine ligand 10; HHC, healthy household contact; IFNγ, interferon-gamma; IGRA, IFNγ release assays; IP-10, IFNγ-inducible protein 10; LID, leprosy IDRI diagnostic; LL, lepromatous leprosy; MB, multibacillary; MDT, multidrug therapy; MLCS, *M. leprae* cell sonicate; OD, optical density; PB, paucibacillary; PGL, phenolic glycolipid; PHA, phytohemagglutinin; PPD, *M. tuberculosis* purified protein derivative; PPV, positive predictive value; RIF, rifampicin; ROC, receiver operating curve; NPV, negative predictive value; NT-P-BSA, semi-synthetic trisaccharide of the PGL-I antigen linked to BSA; TB, tuberculosis; TT, tuberculoid; WBA, whole blood assay; WHO, World Health Organization.

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Given the dichotomous immune responses observed during leprosy, immunological tests are regularly proposed, but only occasionally used, to complement clinical diagnosis. Tests detecting antibodies against *M. leprae* antigens, such as phenolic glycolipid (PGL)-I and leprosy IDRI diagnostic (LID)-1, are positive in most MB patients (Duthie et al., 2007; Oskam et al., 2003). More often than not, however, PB leprosy patients are negative in antibody detection tests and T cell–based assays appear more suitable for the detection of PB cases (Duthie, Ireton, et al., 2008; Geluk, 2013; Sampaio et al., 2012). IFNγ release assays (IGRA) such as QuantiFERON-TB-Gold-In-Tube® (Qiagen, Chadstone, Australia) and the T-SPOT® tuberculosis (TB) assay (Oxford Immunotec, Abingdon, United Kingdom) have been commercialized for the diagnosis of *M. tuberculosis* infection. To advance toward similar tests for leprosy, our group and others have screened a large number of *M. leprae* antigens and have identified several candidates that can elicit IFNγ secretion upon incubation with cells or blood from PB leprosy patients (Duthie, Goto, et al., 2008; Geluk, 2013; Geluk et al., 2008; Geluk et al., 2009; Geluk et al., 2011; Sampaio et al., 2011; Sampaio et al., 2012). Recent work from our group identified that combinations of ML0276 + LID-1 and 46f + LID-1 synergize to enhance IFNγ secretion upon incubation with blood from PB leprosy patients, indicating that these combinations hold promise for the further development of IGRA for PB leprosy (Oliveira et al., 2014).

While antigens that stimulate IFNγ release have been able to distinguish PB patients from MB patients and healthy individuals among the general population, to date, antigens or assay readouts that can reliably distinguish PB patients from healthy household contacts (HHCs) of leprosy patients have not been identified. It is generally assumed that responses in HHC arise because these individuals are either continuously exposed to and primed with *M. leprae* antigens or are actually infected with *M. leprae* but lack overt clinical symptoms (Bobosha et al., 2012; Martins et al., 2012; Sampaio et al., 2011). Antigen-specific IGRA developed for TB similarly cannot differentiate active disease from latent diagnosis of *M. tuberculosis* infection (Barth et al., 2008; Moon and Hur, 2013). Measurement of C-X-C motif chemokine ligand 10 (CXCL10; also known as IFNγ-inducible protein 10) has, however, been indicated to distinguish active from latent TB (Clifford et al., 2015; Hong et al., 2012; Wergeland et al., 2015). Given this finding, to evaluate the potential to differentiate active PB leprosy from asymptomatic *M. leprae* infection (HHC), we measured both IFNγ and CXCL10 release in whole blood assays (WBA) using combinations of *M. leprae* antigens.

2. Materials and methods

2.1. Study participants

Recruitment of patients was conducted in 2 areas of medium endemicity for leprosy (1.0–4.9 cases/10,000 inhabitants) in Brazil: a) Goiânia, Goiás State, 1.4 million inhabitants, Central-West region, 2.71 leprosy cases/10,000 inhabitants; and b) Fortaleza, Ceará State, 2.5 million inhabitants, Northeast region, 1.96 leprosy cases/10,000 inhabitants (BRASIL, 2013). In Goiânia, leprosy patients were enrolled at 2 public health outpatient clinics ("Hospital das Clínicas, Universidade Federal de Goiás", and "Centro de Atendimento Integral a Saúde-Finsocial"), while in Fortaleza, patients were enrolled at "Centro de Dermatologia Dona Libânia/CDERM". This study was approved by the pertinent local review committee overseeing each site ("Hospital das Clínicas da Universidade Federal de Goiás" and "Centro de Dermatologia Dona Libânia/CDERM", protocols #456.226, #689.445, and #931.511).

A total of 156 participants, from both genders and all older than 18 years, were recruited. Individuals were excluded if they were under 18 years old, pregnant, or had comorbidities such as AIDS, TB, and diabetes mellitus. Most study participants (65%) had a scar resulting from neonatal bacille Calmette-Guerin vaccination. Leprosy patients were classified by WHO criteria as MB or PB, but were also further categorized based on clinical, bacteriological, and histopathological data into Ridley Jopling classification (Ridley and Jopling, 1966). PB leprosy included TT/BT forms presenting up to 5 skin lesions and low/negative BI (0–0.2). MB patients comprised BB, BL, and LL forms with more than 5 leprosy skin lesions and positive BI. Four study groups were evaluated after undergoing thorough clinical examination to ensure appropriate classification as: 1) newly diagnosed, untreated PB (n = 38: Fortaleza n = 26, Goiânia n = 12; 10 TT, 28 BT); 2) newly diagnosed, untreated MB (n = 30: Fortaleza n = 13, Goiânia n = 17; 21 BL, 9 LL); 3) HHC, defined as adults living in the same house as an MB index case for at least 6 months (n = 27: Fortaleza n = 13, Goiânia n = 14), and 4) endemic controls (EC), defined as individuals living in the same endemic settings but without previous diagnosis of leprosy or TB and having no known contact with leprosy patients. All endemic controls were physically evaluated and no suspected leprosy lesions were found (n = 61: Fortaleza n = 13, Goiânia n = 48) (Table 1).

2.2. Recombinant proteins

M. leprae genes were cloned into *Escherichia coli* and recombinant proteins purified as previously described (Duthie, Goto, et al., 2008). Endotoxin level of each protein was <100 units/mg protein as determined by limulus amebocyte lysate QCL-1000 assay (Lonza, Basel, Switzerland). Two combinations of antigens were evaluated: 46f + LID-1 and ML0276 + LID-1. 46f is a diffusion protein generated by tandem linkage of the *ml*0405 and *ml*0568 genes. LID-1 is a diffusion protein generated by tandem linkage of the *ml*0405 and *ml*2331 genes. These individual proteins and the antigen combinations were previously shown to be immunogenic and capable of eliciting a specific cellular immune response (Oliveira et al., 2014; Sampaio et al., 2011).

2.3. Whole blood assay

Whole blood assays were performed with slight modifications from the previously described methods (Duthie, Goto, et al., 2008). Venous undiluted blood (450 μL/tube) was incubated in individual heparinized tubes (instead of 24-well plates), 37 °C at 5% Co_2 with previously described antigen combinations at a final concentration of 10 μg/mL or controls (*M. leprae* cell sonicate [MLCS], *M. tuberculosis* purified protein derivative [PPD], and phytohemagglutinin [PHA; Sigma], all at 10 μg/mL final). PHA was included as an assay positive control to confirm that there were cells present in the WBA capable of responding to a T-cell stimulant. PBS was included as a negative control to demonstrate that the levels of IFNγ/CXCL10 measured were not due to ex vivo production. PPD was included as a general mycobacterial antigen control and MLCS, a *M. leprae* crude antigen mix, was used as *M. leprae*–directed control. After incubating for 24 hours, 150 μL of plasma was collected from each tube and frozen at −20 °C until further analysis.

2.4. Cytokine ELISA

IFNγ content was measured by QuantiFERON enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (QuantiFERON-TB Gold/QFT-G®; Qiagen, Carnegie, Australia). Results were converted to pg/mL on the manufacturer's instructions that 1 IU/mL of IFNγ is equivalent to 40 pg/mL. As previously described, an arbitrary threshold of 50 pg/mL was adopted to define positive responses (Oliveira et al., 2014).

CXCL10 levels were measured by Human CXCL10 ELISA Kit, according to the manufacturer's instructions (Sigma-Aldrich®, St Louis, MO, USA). The threshold for positive responses was determined by receiver operating characteristic (ROC) curve analysis and a threshold of 500 pg/mL for WBA using 46f + LID-1 and ML0276 + LID-1 was adopted.