Diagnostic Microbiology and Infectious Disease 83 (2015) 154–161

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

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

Alterations to antigen-specific immune responses before and after multidrug therapy of leprosy

Aline Araújo Freitas [a], Regiane Morillas Oliveira [a], Emerith Mayra Hungria [a], Ludimila Paula Vaz Cardoso [a], Ana Lúcia Osório Maroccolo Sousa [a], Maurício Barcelos Costa [a], Steven G. Reed [b], Malcolm S. Duthie [b], Mariane Martins Araújo Stefani [a,*]

[a] Tropical Pathology and Public Health Institute, Federal University of Goias, Goiania, Brazil
[b] Infectious Disease Research Institute, Seattle, WA, USA

ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form 23 June 2015
Accepted 29 June 2015
Available online 2 July 2015

Keywords:
Leprosy
Diagnosis
Serology
Cellular immunity
Multidrug therapy

ABSTRACT

This study evaluated the impact of leprosy multidrug therapy (MDT) on cell-mediated immunity (CMI) and antibody responses at diagnosis in untreated paucibacillary (PB) (n = 15) and multibacillary (MB) patients (n = 15) using a panel of *Mycobacterium leprae* recombinant antigens (rMLs) (CMI: 46f, ML0276, ML2055, leprosy IDRI diagnostic 1 [LID-1], and ML2629, as negative control; serology: LID-1, 46f, 92f, and 33f, as negative control, and phenolic glycolipid I [PGL-I]) and at 2 time points after MDT (PB: 8–20 months; MB: 4–22 months). At diagnosis, PB patients produced interferon gamma (IFNγ), and MB patients exhibited low/absent response. Shortly after MDT, IFNγ production in PB patients declined except for LID-1; MB patients produced IFNγ to LID-1. Almost 2 years after MDT, IFNγ levels declined in PB and MB patients. Most untreated PB patients were seronegative to PGL-I and rML, remaining so after MDT. Most untreated MB patients were seropositive to all antigens, and IgG to rMLs declined after MDT. Reduction in antigen-specific CMI in PB and in antibody response in MB patients may help monitor MDT effectiveness.

1. Introduction

Leprosy is a chronic disease caused by *Mycobacterium leprae* that can cause irreversible peripheral nerve damage (Lockwood, 2002). Despite the effectiveness of multidrug therapy (MDT), leprosy incidence appears not to have changed significantly, and 215,656 newly diagnosed cases were still reported in 2013. Brazil reported 31,044 new leprosy cases in this time period, ranking second in overall leprosy incidence to only India (WHO, 2014).

Leprosy presents a wide and complex spectrum of bacteriologic, clinical, and histopathologic signs that are dependent on the host immune response. Paucibacillary (PB) leprosy patients present absent bacterial indices (BI), few localized skin and neurological lesions, strong specific cell-mediated immunity (CMI), and low/absent antibody production. Multibacillary (MB) disease is characterized by high BI, multiple disseminated skin lesions, weak/absent *M. leprae*–specific CMI, and high titers of specific antibodies (Scollard et al., 2006). MDT currently consists of 6 doses with rifampicin and dapsone for PB leprosy and 12 doses with rifampicin, dapsone, and clofazimine for MB patients. Rifampicin is taken once monthly, but the other drugs are scheduled differently (WHO, 2009). Upon MDT completion, patients are considered cured; however, even after MDT, some patients develop leprosy reactions (www.who.int/lep, 2014). Early diagnosis and treatment before the onset of clinical manifestations are considered key to reduce *M. leprae* transmission and to prevent deformities and disabilities (Scollard, 2008; WHO, 2013).

Currently, the diagnosis of leprosy relies on the recognition of the clinical manifestations. Spurred by the decoding of the *M. leprae* genome, research efforts have pushed toward the identification of *M. leprae* proteins suitable for the development of appropriate laboratory tests. For serology-based tests, the leprosy IDRI diagnostic 1 (LID-1) fusion protein (combining ML0405 and ML2331 in a single product) has been well recognized by IgG antibodies of MB patients from multiple leprosy-endemic areas (Duthie et al., 2008b). Our recent data demonstrated that LID-1 can also induce IFNγ production in whole blood assays (WBAs) of PB leprosy patients and healthy household contacts regularly exposed to *M. leprae* (Oliveira et al., 2014).

While diagnostic and prognostic tests are needed to monitor preventive and therapeutic strategies for leprosy, the impact of MDT on *M. leprae*–specific cell-mediated immune responses is unknown. This study evaluated the impact of MDT on the serological and cellular immune responses against a panel of *M. leprae* recombinant antigens (rMLs).

2. Patients and methods

2.1. Study groups

This longitudinal and prospective study evaluated newly diagnosed, untreated leprosy patients at the time of diagnosis (pre-MDT group)

* Corresponding author. Tel.: +55-62-3209-6111; fax: +55-62-3209-6363.
E-mail address: mmastefani@gmail.com (M.M.A. Stefani).

http://dx.doi.org/10.1016/j.diagmicrobio.2015.06.021
0732-8893/

and at 2 time points after completing MDT (post-MDT groups—8 and 20 months for PB patients and 4 and 22 months for MB patients).

Thirty newly diagnosed, untreated PB and MB leprosy patients were recruited between July 2010 and November 2012 at the public health center for leprosy diagnosis and treatment (Centro de Referência em Diagnóstico e Terapêutica/CRDT in Goiânia, Goiás) in a highly endemic area for leprosy in central western Brazil. This study was approved by the local review board "Comitê de Ética em Pesquisa Humana–Hospital das Clínicas da Universidade Federal de Goiás" protocol number 456.226, and all patients signed an informed consent form. Criteria for inclusion were newly diagnosed, untreated adult (>18 years of age) leprosy patients from either gender. Exclusion criteria included pregnancy and comorbidities such as HIV/AIDS, tuberculosis, diabetes mellitus, or other chronic disease. The same exclusion criteria were also applied to healthy controls who were interviewed and asked about preexisting comorbidities. Each patient was submitted to complete dermatoneurological examination by 1 dermatologist with expertise in leprosy diagnosis. Skin biopsies were obtained from the edges of active skin lesions, and venous blood was collected from all patients.

Patients were classified according to the criteria of Ridley and Jopling (1966) considering histopathologic, bacilloscopic, and clinical findings. For data analysis, tuberculoid (TT) and borderline–tuberculoid (BT) leprosy patients were merged as PB leprosy, and borderline–lepromatous (BL) and lepromatous (LL) patients were considered MB leprosy. All PB leprosy patients (5 TT and 10 BT) had negative bacilloscopy, while MB (11 BL and 4 LL) leprosy patients had a mean BI of 3.0 (range 1.25–3.25). The median ages were 54 years for PB leprosy patients (range 31–71 years; SD 13) and 51 years for MB leprosy patients (range 23–73 years; SD 13). The following male/female ratio was reported for each group: 6:9 in PB leprosy and 11:4 among MB leprosy patients. Most study participants (96%) had a scar associated with neonatal Bacille Calmette Guerin vaccination.

2.2. Post-MDT groups

All enrolled patients were invited to return at predefined time points after completion of MDT. However, for several reasons, patients returned according to their convenience, reflecting different time points after MDT. Besides pre-MDT immunological evaluation, most enrolled patients returned at least twice after MDT: shortly after MDT (4–8 months for MB and PB patients, respectively) and around 2 years after MDT (20 months for PB and 22 months for MB patients). Among the main causes of loss to follow-up of patients, we can list: lack of availability to return due to difficulty in getting time off from work, dissatisfaction with the need for repeated visit and blood collection, and inability to contact the patient either because he/she had changed the telephone number or moved away to an unknown location.

2.2.1. First evaluation post-MDT

Of 30 of newly diagnosed untreated leprosy patients originally recruited, 23 (77%) returned to the reference center after completing the MDT between July 2012 and July 2013; 7 patients (23%) were lost to follow-up. These 23 patients consisted of 12 MB and 11 PB leprosy patients recruited between 4 and 8 months after the conclusion of MDT.

2.2.2. Second evaluation post-MDT

Approximately 20 months after the conclusion of MDT (22 months for MB and 20 months for PB patients), 18 out of 23 patients (78%) returned to the reference center (9 PB and 9 MB leprosy patients) for a new evaluation of their health status and for blood collection.

2.3. M. leprae recombinant proteins

Cloning and purification of rML were performed as previously described (Duthie et al., 2008b; Reece et al., 2006). The CMI of leprosy patients was evaluated using the following rML: ML0276, ML2055, 46f, LID-1, and ML2629 as negative control. *M. leprae* proteins used in this study were previously evaluated and considered as immunogenic and capable of eliciting specific immune response (Duthie et al., 2008a, 2008b; Oliveira et al., 2014; Sampaio et al., 2011). The humoral response was evaluated by IgG antibodies to rML LID-1, 46f, 92f and 33f (negative control) (Hungria et al., 2012) and IgM antibodies to phenolic glycolipid I (PGL-I) (Bührer-Sekula et al., 1998).

2.4. CMI determination by WBA

WBAs were performed as previously described (Duthie et al., 2008a). Briefly, undiluted, heparinized whole blood was plated (24-well plates; 450 μL/well; Sigma, St Louis, MO, USA) with each individual rML (10 μg/mL; LID-1, 46f, ML0276 and ML2055), phosphate-buffered saline (PBS) alone (background control), and ML2629 (10 μg/mL; negative control); positive controls consisted of *M. leprae* cell sonicate (10 μg/mL; provided by Dr John Spencer, Colorado State University, Fort Collins, CO, USA, under National Institutes of Health contract N01 AI-25469) and phytohemagglutinin (1 μg/mL; Sigma). After incubation (24 h, 37 °C, 5% CO_2), plasma was collected and stored (−20 °C) until tested for human IFNγ (QuantiFERON®-TB Gold/QFT-G; Qiagen, Hilden, Germany). The results were converted from IU/mL into pg/mL (1 IU/mL of IFNγ being equivalent to 40 pg/mL according to the manufacturer's instructions). The detection limit was 5 pg/mL, and an arbitrary cutoff point of 50 pg/mL was adopted to determine positive responses.

2.5. Detection of antibodies to M. leprae antigens

Serum IgG antibodies against rMLs and IgM antibodies to *M. leprae*–specific PGL-I were detected by enzyme-linked immunosorbent assay (ELISA). Briefly, for the IgG reactivity, PolySorp 96-well plates (Corning Costar, Corning, NY) were coated with each individual rML (1 μg/mL) and blocked (4 °C, PBS–Tween-20, 1% bovine serum albumin [BSA]). Diluted serum samples (1/200 in 0.1% BSA) were incubated in duplicate (2 h, room temperature); plates were washed and incubated with horseradish peroxidase (HRP)–conjugated anti-human IgG (Southern Biotech, Birmingham, AL, USA). After washing, reactions were developed (peroxidase color substrate; KPL, Gaithersburg, MD, USA) and quenched (1 N H_2SO_4). The corrected optical density (OD) of each well at 450 nm was determined using a Multiskan Ex microplate reader (Thermo Scientific, San Jose, CA, USA). The threshold for positive responses was previously calculated (2× SD of the OD of sera from healthy endemic controls), so the samples with OD >0.3 were considered positive as described in Duthie et al. (2007). The results are expressed as the mean OD of duplicates.

For IgM reactivity to PGL-I, ELISAs employed coated plates with 0.01 mg/mL of the natural trisaccharide-phenyl synthetic analog of PGL-I conjugated to BSA (NT-P-BSA) (kindly provided by Dr Fujiwara, Nara University, Japan; PolySorp 96-well plates; Nunc, Roskilde, Denmark). Blocking was performed with 1% BSA/PBS. Diluted serum samples (1/300 PBS-BSA, 10% normal goat serum; Sigma-Aldrich, St Louis, MO, USA) were tested with either NT-P-BSA or BSA-coated wells. After incubation and washing, HRP-conjugated anti-human IgM (Immuno Chemicals, St Louis, MO, USA) was added, incubated, and washed; 3,3′,5,5′-tetramethylbenzidine liquid substrate (Sigma-Aldrich) was added, and the reaction was quenched (2.5 N H_2SO_4; Sigma-Aldrich). To control intratest and intertest variations, a duplicate of positive reference serum was used on each plate, and reactions stopped when its reading reached an OD of 0.6. The OD was measured at 450 nm (Bio-Rad microplate reader; Life Science, Hercules, CA, USA). The final OD was calculated by subtracting the OD BSA-coated wells from OD values of NT-P-BSA wells. The cutoff was defined as OD > 0.25 in accordance with Bührer-Sekula et al. (1998).