HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND MYCOBACTERIUM LEPRAE CO-INFECTION: HIV-1 SUBTYPES AND CLINICAL, IMMUNOLOGIC, AND HISTOPATHOLOGIC PROFILES IN A BRAZILIAN COHORT

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Abstract. Co-infections with human immunodeficiency virus (HIV) and Mycobacterium leprae represent unique opportunities to investigate the interaction of both pathogens. We determined the immunologic, virologic, and histopathologic characteristics of 22 co-infected Brazilian patients (median age = 38 years, 81.8% males, 72.2% with paucibacillary leprosy, and 95.4% with acquired immunodeficiency syndrome). The HIV-1 subtypes B and BF predominated in envelope and gag heteroduplex mobility analysis. Borderline tuberculoid (BT), tuberculoid, lepromatous, and indeterminate morphology with CD3+, CD8+, and CD68+ cell distributions compatible with leprosy patients not infected with HIV were observed. Histologic evidence of nerve damage was observed in BT lesions. IgM antibody to M. leprae-specific phenolic glycolipid I was not detected. Two of six co-infected patients monitored during highly active antiretroviral therapy (HAART) developed a leprosy type 1 reaction after an increase in CD4+ cells, suggesting an immune restoration phenomenon. Clinical, immunologic, histopathologic, and virologic features among these HIV-leprosy co-infected patients indicate that each disease progressed as in single infection. However, HAART immune reconstitution may trigger potential adverse effects, such as leprosy acute inflammatory episodes.

INTRODUCTION

A well known consequence of infection with human immunodeficiency virus type 1 (HIV-1) is higher susceptibility to opportunistic mycobacterial infections, especially with the Mycobacterium avium complex and M. tuberculosis, both of which are correlated with increased morbidity and mortality.1,2 During the first two decades of acquired immunodeficiency syndrome (AIDS) pandemic, HIV has spread throughout tropical and subtropical areas endemic for infection with M. leprae, such as Brazil and many African countries, and has resulted in the investigation of possible adverse interactive effects.3 The impact of several co-infections on the pathogenesis of HIV-1 infection in Africa has been recently reviewed.4 Cellular immunity mediated by CD4+ T lymphocytes, which gradually disappear in HIV-1 infection, constitutes an important protective mechanism against both HIV and M. leprae.5,6 The spectrum of the clinical forms of leprosy depends on the CD4+ Th1 cellular immune responses to M. leprae, ranging from immunocompetent localized tuberculoid paucibacillary (PB) forms to anergic disseminated multibacillary lepromatous (LL) cases.6,7 The unique M. leprae tropism for Schwann cells of the peripheral nerves can cause intra-neural inflammation in any form of the spectrum that if not treated may potentially lead to impairments and permanent disability.8 During the course of leprosy, before or long after the specific antibiotic use, patients can develop acute inflammatory episodes known as a type 1 reaction (T1R) and erythema nodosum leprosum (ENL). These episodes are considered major complications that can cause nerve damage and short-term and long-term morbidity.

In dually infected patients, as immunosuppression induced by HIV-1 becomes apparent, unfavorable leprosy outcomes should be expected. A higher incidence of multibacillary (MB) cases, an increase in transmission, and a higher frequency of reactions especially, ENL were initially expected. However, several studies have indicated that in individuals dually infected with leprosy and HIV, one condition does not predispose the occurrence or worsening of the other.3,9-13 As of December 2001, there were more than 236,000 cases of AIDS and an estimated 600,000 HIV-positive individuals in Brazil, which is the only Latin American country where leprosy is still endemic.14,15 While evidence of genetic variability in M. leprae is scarce, HIV-1 shows remarkable genetic variability.16,17 Group M of HIV-1 is responsible for the global AIDS pandemic and is composed of nine subtypes (A, B, C, D, F, G, H, J, and K) and 15 inter-subtype recombinant forms co-circulating in many areas of the world.18 Although the clinical implications of HIV-1 diversity are not well known, especially in co-infected patients, HIV diversity has been shown to be important in detection assays, resistance monitoring, development of resistance to antiretroviral agents, and vaccine design.17

The recent availability of potent combinations of antiretroviral agents (two nucleoside and one non-nucleoside reverse transcriptase inhibitor or nucleoside reverse transcriptase inhibitors and a protease inhibitor) known as highly active antiretroviral therapy (HAART) has resulted in immune reconstitution, viral load reductions, and dramatic decreases in HIV-related morbidity and mortality.19 However, restoration of inflammatory responses has been shown to trigger overt clinical manifestation of co-infection with M. tuberculosis, nontuberculous mycobacteria, cytomegalovirus, hepatitis B and C viruses, and more recently, with M. leprae.20,21 In Brazil, the use of HAART increased in the late 1990s, and it is currently being offered at no cost to AIDS patients by the Ministry of Health. The successful implementation of multidrug therapy (MDT, rifampin, dapsone, and clofazimine) for leprosy has contributed to a dramatic decrease in its prevalence in endemic countries, and is an important component of the elimination strategy advocated by the World Health Organization. Nevertheless, despite almost 10 years of high MDT coverage in Brazil, 42,055 incident cases of leprosy were reported in 2000.14,22

The current situation concerning leprosy endemcity and HIV-1 prevalence in Brazil and in other developing countries emphasizes the importance of monitoring co-infections. In
MATERIALS AND METHODS

Study population. The study group in central Brazil was composed of HIV- M. leprae co-infected patients in Goiânia (population ~ 1,200,000), the capital of Goiás State (population ~ 4,000,000), which is located 200 km from Brasília, the Federal Capital. In this setting, independent public health programs are responsible for HIV and leprosy diagnosis, treatment, management, and control. Leprosy patients are assisted at the State Secretariat of Health and HIV-positive and AIDS patients are diagnosed, treated, and monitored at the main regional public reference hospital for infectious diseases (Anuar Auad Hospital [HAA/HDT/SUS]), where HIV- M. leprae co-infected patients were identified by a careful review of the databank of HIV-infected patients. Co-infected patients included both HIV-1-positive patients who were simultaneously (within a six-month interval) or subsequently diagnosed with leprosy and leprosy patients subsequently diagnosed as being infected with HIV-1.

A retrospective assessment of all regional cases of HIV- M. leprae co-infection was performed based on a careful investigation of case report forms from all HIV/AIDS recorded cases at HAA/HDT/SUS. A standardized questionnaire was used to record sex, age, CD4 cell and viral load values, residence, and behavioral risk to HIV infection, including sexual activity, intravenous drug use, and blood transfusions. Data regarding peripheral CD4+ T cell counts (Fluorescence Activated Cell Sorter; Becton Dickinson, San Jose, CA) and viral loads (Nucleic Acid Sequence Based Amplification® NucliSens™ kits; Organon Teknika, Boxtel, The Netherlands) were obtained from medical files.

For the investigation of immunologic and virologic profiles, a subgroup of HIV- M. leprae co-infected patients was recruited from August 1997 to July 2000 independent of their leprosy MDT and antiretroviral treatment. Before collection of biologic specimens, patients were given a detailed clinical-dermatologic examination by dermatologists with expertise in leprosy and by specialists in infectious diseases. Data regarding clinical evolution were obtained from medical files and updated as of April 2004. For treatment purposes, patients were clinically classified as PB and MB leprosy. Classification criteria for leprosy and for HIV infection/AIDS were revised according to guidelines established by the Brazilian Ministry of Health.

Blood samples were collected from all participants into tubes containing EDTA and biopsies were performed on patients with leprosy skin lesions. Leprosy skin biopsy specimens were collected after a diagnosis of HIV/leprosy. Blood-EDTA samples were used for HIV-1 genetic characterization and plasma was used for serologic analysis of antibody to M. leprae phenolic glycolipid I (PGL I). Leprosy skin biopsies were used for the histopathologic classification of nerve damage whenever observed. Fite-Faraco staining for detection of bacilli and cell phenotyping by immunostaining were performed.

This study was reviewed and approved by the local ethical review board (Comitê de Ética em Pesquisa Humana do HAA/HDT/SUS). Written informed consent was obtained from all participants in the immunologic and virologic studies.

Multibacillary leprosy cases were defined by clinical signs compatible with borderline lepromatous (BL) or LL leprosy or a patient with a positive bacterial index (BI) of the skin smears and/or any leprosy patient with more than six skin lesions. Paucibacillary leprosy cases presented clinical signs compatible with tuberculoid (TT) or borderline tuberculoid (BT) leprosy and with a negative BI of the skin smears and/or any leprosy patient with a negative skin smear or presenting up to five skin lesions. Paucibacillary leprosy patients were treated with 6–9 months of leprosy MDT and MB patients were treated with 12–18 months of leprosy MDT treatment according to Brazilian Ministry of Health guidelines. A T1R was defined as the appearance of erythema and edema in either existing or new leprosy skin lesions with or without neuritis, pain, and/or tenderness of the involved peripheral nerves, as evaluated by trained physicians. The T1R was treated for 32 weeks with corticosteroids (1–2 mg/kg/day). Acquired immunodeficiency syndrome cases were defined by a modified criteria adopted by the Brazilian Ministry of Health that includes patients with CD4 cell counts less than 350 cells/mL or clinical conditions related to AIDS.23

Genetic characterization of HIV-1. Whole blood-EDTA stored at −70°C, was depleted of red blood cells by treatment with 1 M Tris-HCl, 1 M sucrose 1 M MgCl2, 1% Triton X-100, and genomic DNA was extracted from peripheral blood mononuclear cell lysates with DNAzol (Molecular Research Center Inc., Cincinnati, OH). The DNA was precipitated with ethanol, resuspended in Tris-EDTA buffer, and stored at −70°C. The HIV-1 envelope (env) and gag genes were amplified by a nested polymerase chain reaction using the primer pairs ED5/ED12 and ES7/ES8 for the env gene and H1P202/H1G777 and H1Gag1584/g17 for the gag gene. Determination of the HIV-1 subtypes on the env and gag genes was performed by heteroduplex mobility analysis (HMA) using reagents (HMA HIV-1 env subtyping kit, protocol version 5 and HIV-1 gag HMA subtyping kit, protocol version 3) provided by the National Institutes of Health/AIDS Reagent Program (Germantown, MD) (www.aidsreagent.org).24–26

Immunostaining and histopathologic analysis of leprosy skin lesions. Leprosy skin biopsy specimens (4 mm) from HIV- M. leprae patients were used for immunostaining, conventional histopathologic staining with hematoxylin and eosin, and Fite-Faraco staining for acid-fast bacilli (AFB). Whenever applicable, the Ridley-Jopling histopathologic classification criteria for leprosy were used. For immunohistochemical analysis, rehydrated deparaffinized tissue sections were treated to block endogenous peroxidase, pressure cooker antigen retrieval was performed, and non-specific protein binding was blocked by incubation with fat-free bovine milk. Tissue sections were incubated with the following cell-specific primary antibodies: monoclonal antibody (MAb) to CD68+ macrophages (1:20,000 dilution; Novocastra, Newcastle upon Tyne, United Kingdom), polyclonal antibody to CD3+ T cells (1:4,500 dilution; Dako, Carpinteria, CA), MAb to CD8+ T cells (1:160 dilution; Novocastra), and MAb to natural killer (NK) CD57+ cells (1:100 dilution; Novocastra).
Isotype-specific secondary biotinylated antibodies were used and the reaction product was detected with an avidin-biotin-peroxidase complex (Novocast). The color reaction was developed with 3,3′-diaminobenzidine chromogen (Merck, Darmstadt, Germany) and counterstained with hematoxylin (Sigma, St. Louis, MO). The presence of a dark brown precipitate visible by light microscopy indicated a positive reaction.

Serologic analysis of antibody to PGL I. Specific IgM antibodies for *M. leprae* PGL I were assayed with an enzyme-linked immunosorbent assay (ELISA) as previously described.²⁷,²⁸ Briefly, 0.25 μg/mL of PGL I synthetic disaccharide (kindly provided by Dr. M. J. Colston, National Institute for Medical Research, London, United Kingdom), serum samples diluted 1:300, peroxidase-conjugated goat anti-human IgM (μ chain specific; Sigma), and 0.4 μg/mL of o-phenylenediamine substrate dye (Sigma) were used. The optical density was read at 492 nm in an ELISA reader and the reaction product was detected with an avidin-biotin-peroxidase complex (Novocastra). The color reaction was developed with 3,3′/H11032 and 0.4/o,3/H11505 g/mL of PGL I synthetic disaccharide and a dark brown precipitate was visible by light microscopy.

Phenotypic analysis of human HIV-1 strains. The phylogenetic analysis of HIV-1 strains was performed with the neighbor-joining method using the ClustalX program and the bootstrap test with 1000 replicates. The ATG codon was deleted to avoid any bias. The deduced amino acid sequences were aligned and ambiguous sites were eliminated from the alignment. The sequences were analyzed with the Phylip (Department of Microbiology, University of Oslo, Norway) and the PAML (Laboratory for Molecular Evolution, University of Oslo, Norway) programs.

RESULTS

Characteristics of the HIV- and *M. leprae* co-infected patients from central Brazil. Since the beginning of the AIDS pandemic, 34 patients co-infected with HIV and *M. leprae* were diagnosed at HAA/HDT/SUS. The median age of these patients was 36 years (range = 25–55 years) and most (73.5%) was urban residents. The male:female ratio was 24:10 and 70.58% had clinical diagnosis of paucibacillary leprosy. At the time of the diagnosis of HIV- and *M. leprae* co-infection, 94.11% were considered to be AIDS cases. Among the 34 co-infections 14 (41.18%) were first diagnosed with HIV, 10 (29.41%) were first diagnosed with HIV and then leprosy within a six-month interval, and 10 (29.41%) were first diagnosed with leprosy. Co-infected patients were treated with the appropriate MDT regimen for PB or MB leprosy. The majority (82.3%) of the co-infected patients was diagnosed before the introduction of HAART and were treated with various nucleoside reverse transcriptase inhibitors. Information about the exposure route obtained for 24 patients indicated that sexual route was reported by 21 patients (12 heterosexual, 5 homosexual, and 4 bisexual) and the parenteral route was reported by 3 patients (2 received blood derivatives and 1 was an intravenous drug user). As of December 2000, 5 of 34 dually infected patients had died, mostly due to AIDS complications, without any relationship to leprosy (2 of hypovolemic shock, 1 of *Pneumocystis carinii* pneumonia, 1 of sepsis, and 1 of neurologic disease of unknown etiology). One patient died of cirrhosis during this study and seven patients were considered lost to follow-up.

Virologic, clinical, and immunologic characteristics. Table 1 summarizes main immunologic and virologic characteristics of 22 HIV- and *M. leprae* co-infected patients, including HIV-1 subtypes detected by HMA. The HIV-1 subtype B in the env and gag regions predominated in this cohort. In the env region, 16 of 18 samples had HIV-1 subtype B and 2 had the BF profile. In the gag region, subtype B was detected in 15 samples and subtype F was detected in two samples.

Among this subgroup of dually infected patients, the majority (15 of 22) was diagnosed before the introduction of HAART. Seven co-infected patients were diagnosed simultaneously for HIV and leprosy, six of them (patients 23, 26, 29, 31, 32, and 34) after 1998 when HAART was prescribed (Figure 1). They began treatment with HAART, followed a short time later by leprosy MDT, and most of them had significant increases in the CD4 counts. Two of them (patients 29

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<th>Viral load (copies/mL)</th>
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* AFB = acid-fast bacilli; AIDs = acquired immunodeficiency syndrome; PB = paucibacillary; MDT = multidrug therapy; RTN = reverse transcriptase nucleoside inhibitor; MB = multibacillary; LL = lepromatous leprosy, Nai = naive/untreated; BT = borderline tuberculoid; I = indeterminate; TT = tuberculoid; HAART = highly active antiretroviral therapy.

† HIV-1 subtype not identified by heteroduplex mobility analysis.

‡ Patients who developed a type I reaction after HAART.
and 32) presented 2–6 months after beginning HAART with an acute leprosy inflammatory episode known as a T1R. Following HAART, the CD4 counts had increased from 73 to 270 cells/μL (a 3.4-fold increase) in one patient and from 35 to 100 cells/μL (a 2.9-fold increase) in another patient. In the four remaining co-infected patients receiving HAART who did not have a T1R, smaller increases in CD4 counts (ranging from 1.2 to 1.8 fold) were observed. Up to the time of this report (April 2004) no other episode of T1R was observed among these co-infected patients and two other deaths related to AIDS were reported (patients 13 and 26). Eight deaths, mostly related to AIDS, were observed among the 34 dually infected patients monitored.

Independent of the antiretroviral regimen in 68.1% (15 of 22) of the dually infected patients, the first CD4 T count measured was less than 200 cells/μL, indicating advanced disease. Viral loads >160,000 copies/mL were detected in 9 of 22 patients (Table 1).

**Histomorphology of leprosy skin lesions.** The microanatomy, distinct cell phenotypes, presence of bacilli, and degree of nerve damage in 18 leprosy skin lesions were assessed by conventional histopathologic analysis and immunostaining (Figures 2A-C). For the purpose of histopathologic assessment of leprosy skin lesions, patients were categorized into three groups according to leprosy MDT status when the skin biopsy specimens were obtained: A) MDT naive (n = 9), B) receiving MDT (n = 7), and C) post-MDT (n = 2).

**Group A.** The histopathologic classification of the nine skin biopsy specimens collected prior to MDT were BT (n = 6), TT (n = 2), and LL (n = 1) leprosy. The BT lesions were characterized by less circumscribed epithelioid granulomas and a few Langerhans CD68+ cells in the center surrounded by T CD3+ lymphocytes (Figure 2A), a few of them with the CD8+ phenotype. Nerve damage was observed in the histopathologic examination of five of six BT lesions and two were AFB positive. The TT leprosy lesions had well formed granulomas with CD68+, epithelioid, multinucleated Langerhans cells and macrophages in the center surrounded by a lymphocytic mantle of CD3+ T cells (Figure 2B) and few CD8+ T cells. The TT lesions were AFB negative and nerves were not visualized. The only leprosy skin lesion categorized as LL had a diffuse CD68+, vacuolated, histiocytic cell infiltrate with few CD3+ lymphocytes (Figure 2C), a high (3+) AFB positivity, and nerve damage. In all histopathologic categories, few NK CD57+ cells were observed.

**Group B.** The histopathology and immunostaining of seven skin lesions (TT = 3, BT = 3, and indeterminate [I] = 1) collected from co-infected patients undergoing leprosy MDT had similar characteristics to the TT, BT, and I lesions of naive co-infected patients, except for a lower level of cellularity.

**Group C.** Two residual leprosy skin lesions collected post-MDT had discreet perivascular/perinanexial indeterminate inflammatory infiltrates and were AFB negative.

**Serologic analysis of antibody to** M. leprae **PGL I.** In this cohort, regardless of the clinical form of leprosy (MB or PB), histopathologic classification, AFB positivity in lesions and MDT status, none of 22 dually infected patients, including one lepromatous case, had detectable IgM antibodies specific for M. leprae PGL I.

### DISCUSSION

This study provided the opportunity to study a large number of Brazilian leprosy patients with concomitant HIV-1 in-
fection. This cohort of well-characterized dually infected patients showed several features of interest. Among HIV-M. leprae co-infected patients, the investigation of HIV-1 subtypes in two different genetic regions (env and gag) showed the predominance of HIV-1 subtype B. These data are compatible with HIV-1 diversity among HIV/AIDS Brazilian patients not infected with M. leprae. In HIV-1 leprosy co-infection, there is no evidence indicating that different HIV-1 subtypes would have any correlation with the development of leprosy or any specific type of leprosy. In Brazil, HIV-1 subtype B predominates among sexual transmission cases and most of the co-infected patients in our cohort reported either a heterosexual or homosexual transmission route.

Among dually infected patients treated with HAART, some presented with an acute leprosy inflammatory episode known as T1R following immune restoration. A significant percentage of the co-infected patients in our cohort was classified as BT, which is considered an immunologically unstable form of leprosy. Within most BT lesions from naive patients, evident histopathologic signs of nerve damage were seen. Type 1 reactions are known to occur in approximately 30% of patients with BT leprosy, accounting for substantial morbidity, hospitalization, and difficulty in clinical management. Among dually infected patients treated with HAART, a T1R in a borderline leprosy patient was described in an HIV patient shortly after the beginning of HAART, suggesting a possible immune reconstitution phenomenon. Our study was not designed to address the causal effect of immune restoration by HAART on T1Rs. However, our findings of two co-infected patients with T1R with a temporal association with HAART may suggest that this inflammatory event could have been triggered by immune reconstitution inflammatory phenomenon. Whether acute inflammatory events are more frequent among dually infected patients following potent antiretroviral regimens requires further investigation.

The immunologic, virologic, and histopathologic profiles of this cohort of dually infected patients support several previous studies indicating a lack of impact of one disease on the other. Our results corroborate earlier reports that the histopathoarchitecture and cell phenotypes within the leprosy skin lesions are not changed by HIV. The microanatomy of leprosy skin lesions in patients co-infected with HIV did not differ from the classic histomorphologic spectrum of leprosy. The identification of CD4+ cells, which was not feasible using antibodies to CD4 cells from two different suppliers on deparaffinized sections, may provide important information about local immune response to M. leprae in dually infected patients. Our findings of well-formed granulomas among severely immunosuppressed AIDS patients support previous studies suggesting that local immunologic mechanisms required for granulomas formation in leprosy skin lesions do not seem to be affected by peripheral immunosuppression observed in AIDS patients.

In leprosy patients, IgM antibody titers to M. leprae-specific PGL I antigen are considered surrogate markers of the bacillary load. Among dually infected patients studied, there was a clear predominance of AIDS-PB leprosy. None of the co-infected patients was seropositive for PGL I, a finding consistent with the low sensitivity of anti-PGL I serology in patients with PB leprosy. In our cohort, only one co-infected patient was LL. However, in lepromatous patients not infected with HIV, 90% positivity for antibody to PGL I was observed. It would be interesting to investigate the positivity of IgM anti-PGL I serology in AIDS patients with MB LL in a larger group of co-infected patients. Monkeys infected with M. leprae and the simian immunodeficiency virus have been demonstrated not to produce antibodies to PGL I. A study conducted in Cuba showed a seropositivity of 14.9% for PGL I among HIV-positive patients without any clinical sign of leprosy. The significance of these results is unclear.

This cohort may not be representative of the majority of dually infected patients regionally and may have a selection bias for patients with easy access to health care centers in urban settings. In Brazil, HIV transmission is higher in the cities and leprosy also occurs in the poorest urban areas. A recommendation to integrate leprosy control programs with HIV control programs to optimize diagnosis, treatment, and management of patients should be sought.

Co-infection with HIV-1 and M. leprae is a rare event in an area endemic for leprosy and HIV in central Brazil. In this cohort of HIV-1 and M. leprae co-infected patients, the immunologic, virologic, and histopathologic features of the patients were similar to leprosy patients not infected with HIV or non-leprosy HIV-infected patients. Since many studies on this field are case reports or describe a small group of patients, a multi-country cohort study using standardized criteria would be necessary to assess the frequency and severity of leprosy reactions and relapses among HIV-M. leprae co-infected patients in comparison with leprosy cases. Such an investigation could also explore the impact of immune reconstitution by HAART on the development of acute inflammatory episodes of leprosy. The current situation of a longer life expectancy for HIV/AIDS patients due to higher efficacy of antiretroviral treatment and relatively short-term regimens for leprosy makes it an interesting opportunity to explore the immunologic and clinical aspects of the interaction of these two infectious diseases.

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