CASE REPORTS

Case-patient 1. An 18-year-old man was referred to the Leishmaniasis Research and Treatment Center (LRTC) at the University of Gondar Hospital in northwestern Ethiopia for evaluation of abdominal swelling and multiple skin lesions over the face, abdomen and lower extremities. Aside from the skin lesions, the patient had non-bloody diarrhea and left upper quadrant swelling and pain, which began to evolve two years earlier. The patient had never received treatment for VL and tuberculosis (TB), had lost 5 kg of body weight over the preceding 2 weeks, and had a high-grade fever and productive cough accompanied by loss of appetite.

At examination, he was cachectic, tachycardic, tachypnic, and febrile. He had conjunctival pallor and splenomegaly, but the liver was normal in size. There were 3–4 multiple vesicular lesions and clear fluid content over the face (left paranasal area) and ear (Figure 1A). Papulo-nodular skin lesions were densely distributed on the lower abdomen, forearm, and lower extremities (Figure 1B and C). He was positive for HIV a week before admission and had CD4 and CD8 counts of 80 cells/mm³ and 768 cells/mm³, respectively. A large number of Leishmania amastigotes (6+ parasitemic grade; >100 amastigotes/microscopic field) was demonstrated in Giemsa-stained smears of the spleen aspirate and a slit skin smear from lesions of the face and extremities. Parasitemia grading was performed as described by Chulay and Bryceson.

Specimens from the spleen and face/extremities were inoculated separately into biphasic MacNeal, Novy, and Nicole (NNN) medium under aseptic conditions. Results of sputum examination were positive for acid fast bacilli, for which he
Case-patient 2. A 39-year-old man was treated for primary VL in 2006. Subsequently, he was treated for three episodes of VL relapse in 2007, 2008, and 2009. He had been treated with ART, initially stavudine, lamivudine, and efavirenz, for the last two years and four months since his first hospitalization. One month before his current admission to LRTC at the University of Gondar on January 10, 2010 (i.e., after receiving ART for 27 months), ART was replaced with a second-line regimen of tenofovir, abacavir, and lopinavir/ritonavir because his CD4 cell count had not increased. His baseline CD4 cell count at the time of ART initiation was 25 cells/mm$^3$; and subsequent counts were 99 cells/mm$^3$ and 78 cells/mm$^3$ after 12 and 16 months of ART, respectively, and 53 cells/mm$^3$ after 27 months of ART.

The patient came to the LRTC with fever, easy fatigability, and increased left upper quadrant swelling of three months duration. On examination, he was febrile, malnourished, and had a body mass index of 13 kg/m$^2$. He had conjunctival pallor, submandibular lymphadenopathy, and hepatosplenomegaly. He also had nodular skin lesions that were distributed over the lower leg (Figure 2) and shoulder regions. Parasitologic examination of spleen aspirates and scrapings from skin lesions showed high parasitic load (5+ parasitaemic grade: 10–100 amastigotes/microscopic field). Specimens from the spleen and skin were inoculated separately into NNN media under aseptic conditions.

With a diagnostic impression of a fourth relapse of VL and cutaneous involvement, he was treated with SSG, 20 mg/kg, and ART was continued. After 17 days of therapy, his clinical condition worsened although the skin lesions decreased significantly. The SSG treatment was discontinued and he was then treated with liposomal amphotericin B (AmBisome®; Gilead Sciences, Dublin, Ireland), 3 mg/kg, in spite of the absence of clinical or laboratory evidence of side effects to SSG. Despite all the efforts, the patient left the hospital against medical advice and his final outcome is not known.

Case-patient 3. A 32-year-old man was diagnosed with World Health Organization stage IV HIV infection (generalized...
lymphadenopathy, oral candidiasis, chronic diarrhea, herpes zoster vesicles) before admission in Tikur Anbessa Specialized Hospital in Addis Ababa, Ethiopia. The patient was receiving prophylaxis for *Pneumocystis jiroveci* pneumonia and ART ( stavudine, lamuvidine, and efavirenz) for 9 months before admission, and had a baseline CD4 cell count of 40 cells/mm³.

When referred to Tikur Anbessa Specialized Hospital, the patient had fever, generalized lymphadenopathy, hepatosplenomegaly, abdominal discomfort, and loss of body weight (7 kg). He also had multiple papular skin lesions over the face (Figure 3), which emerged six months after beginning treatment with ART. The patient had never received treatment for TB and leishmaniasis. Disseminated TB was ruled out by chest radiograph, abdominal sonography for possible intra-abdominal lymphadenopathy, and an acid fast test for mycobacteria. Lymphoma was excluded on the basis of total blood cell counts determined with a bone marrow aspirate. Visceral leishmaniasis and CL were first diagnosed clinically.

Subsequent laboratory investigations confirmed the *Leishmania* infection in bone marrow aspirates by smear (4+ parasitaemic grade: 1–10 parasites/microscopic field), NNN culture, and in skin lesion scrapings (NNN culture). The patient was then treated with Glucantime® (Specia, Paris, France), 20 mg/kg of body weight for 30 days. The CD4 cell count at the time of diagnosis of VL and disseminated cutaneous lesions was 93 cells/mm³. At the end of the treatment, his skin was clear of lesions and he was discharged cured of VL. He had a barely palpable spleen, although there was only a small change in liver size. Both ART and PCP prophylaxis were continued. Assessment of the CD4 cell count after 27 days of treatment with Glucantime® showed a CD4 cell count of 151 cells/mm³. This patient is currently on close follow-up.

MOLECULAR CHARACTERIZATION OF STRAINS

Genomic DNA was extracted from cultured promastigotes of the six isolates obtained from the three patients with HIV/acquired immunodeficiency syndrome (HIV/AIDS) by using the classical phenol-chloroform method. For species identification, the ribosomal DNA internal transcribed spacer 1 (ITS1) was amplified and subjected to restriction fragment length polymorphism (RFLP) analysis with *Hae*III. The PCR-RFLP analysis of ITS1 showed that the species present in viscera and skin lesions of HIV/VL patients was *L. donovani*.

We further investigated the microsatellite profiles of the six isolates across 14 unlinked microsatellite markers. In addition, we included the microsatellite profile of one strain (MOHM/ET/2008/DM-309) isolated from a skin lesion of a patient in Ethiopia with VL/PKDL. For this patient, there was not enough DNA for analysis of the 14 microsatellite loci. Therefore, before microsatellite typing, we performed whole genome amplification for this strain by using the Illustra™ GenomiPhi™V2 DNA Amplification Kit (GE Healthcare, Piscataway, NJ) following the manufacturer’s instructions with slight modifications. As shown in Table 1, the microsatellites profiles of the paired strains obtained from three cases described in this report were identical across all 14 microsatellite loci.

To check the association between a particular parasite genotype and the different disease phenotypes, microsatellites profiles obtained from those seven strains were compared with previously characterized MLMT profiles of 27 *L. donovani* strains isolated from VL cases in Ethiopia (n = 24) and Sudan (n = 3) and of strains isolated from six PKDL patients from Kenya (n = 2) and Sudan (n = 4) (Figure 4). For this analysis, a microsatellite-based distance matrix was generated by using MSA version 3.0. Neighbor-joining trees were then constructed from the resulting matrix by using POPULATIONS version 1.2.28 (http://bioinformatics.org/~tryphon/populations and MEGA version 3.1, with bootstrap values (1,000 replicates) as described.

The 7 strains from Ethiopian HIV/AIDS patients with cutaneous involvement and PKDL strains from other countries in eastern Africa did not belong to a particular *L. donovani* population, subpopulation, or cluster, but were interspersed in the tree (Figure 4). The two strain pairs MHOM/ET/2010/DM-677sk-MHOM/ET/2010/DM-677sp and MHOM/ET/2010/DM-678sk-MHOM/ET/2010/DM-678sp from VL/HIV co-infected patients with concomitant disseminated CL were assigned to one subpopulation (A) of the North Ethiopia/Sudan (NE/SD)
population that comprises VL strains from north Ethiopia and Sudan. The strain pair MHOM/ET/2009/DM-376sp associated with concomitant disseminated cutaneous leishmaniasis was found in the other subpopulation (B) of NE/SD. The isolate MHOM/ET/2008/DM309/skin was different from each one of these subpopulations. The four strains isolated from PKDL cases in Sudan clustered in NE/SD subpopulation A, and the two PKDL strains from Kenya were assigned to the population consisting of VL and PKDL strains from southern Ethiopia and Kenya.

**DISCUSSION**

Infections with protozoan parasites of the genus *Leishmania* can cause a broad spectrum of syndromes, ranging from simple self-healing CL to the most fatal visceral form depending on the parasite species and the immunogenetic background of the infected persons. In the Americas, mainly in Brazil, disseminated CL caused by *L. braziliensis*, *L. guyanensis*, and *L. amazonensis* is an emerging common health problem in non-immunocompromised persons. In severely immunosuppressed patients, such as those with HIV/AIDS, clinical manifestations of *Leishmania* infections can be atypical. Dermatotropic species can visceralize and viscerotropic species can disseminate from reticulo-endothelial sites to the skin and cause disseminated CL. In Ethiopia, three dermatotropic species can disseminate from reticulo-endothelial sites to the skin, all of our patients were diagnosed as cases of VL and disseminated CL.

In HIV/VL co-infected patients who have received ART and showed evidence of immune reconstitution, multiple lesions could also be clinically recognized as PKDL/VL associated with inflammatory syndrome (IRIS). However, until now, there are no clear clinical or laboratory criteria to define IRIS in leishmaniasis patients. Case-patient 1 had never received ART. Thus, the appearance of multiple cutaneous lesions with high parasite load is more likely to be related to a dissemination of hematogenous *L. donovani* parasites because of immune failure. Immunosuppressed patients are not able to mount an efficient T cell response for controlling *Leishmania* infections. Conversely, case-patient 2 had been receiving ART and was treated with SSG for VL but relapsed several times before the first onset of skin lesions. Clinical follow-up of this patient suggested the absence of immune reconstitution after initiation of ART. This finding and identification of high parasite load and genetically identical *L. donovani* in viscera and skin lesions led us to consider this patient as a case of VL with disseminated CL caused by severe immunosuppression (CD4 cell count < 200 cells/mm³).

Case-patient 3 could be considered as a case of VL and disseminated CL or PKDL/VL associated with IRIS. The main reasons for this diagnosis are a low CD4 cell count (< 50 cells/mm³) before initiation of ART, first appearance of skin lesions after six months of ART, and identification of high parasite load and genetically identical *L. donovani* in viscera and skin lesions. However, an increase of CD4 T cells from 40 to 93 cells/mm³ cannot signify an immune reconstitution or even an increase in lymphocyte effector function in this patient. Thus, this patient is most likely a case of VL with disseminated CL caused by severe immunosuppression (CD4 cell count < 200 cells/mm³).

Case-patient 4 could be considered as a case of VL and disseminated CL or PKDL/VL associated with IRIS. The main reasons for this diagnosis are severe immunosuppression (CD4 cell count < 50 cells/mm³) before initiation of ART, first appearance of skin lesions after six months of ART, and identification of high parasite load and genetically identical *L. donovani* in viscera and skin lesions. However, an increase of CD4 T cells from 40 to 93 cells/mm³ cannot signify an immune reconstitution or even an increase in lymphocyte effector function in this patient. Thus, this patient is most likely a case of VL with disseminated CL caused by severe immunosuppression (CD4 cell count < 200 cells/mm³).

In our patients, *L. donovani* were identified in specimens obtained from skin lesions and viscera. Molecular characterization by MLMT of *L. donovani* parasites from these two body sites showed that they were genetically identical. Multiple lesions of our patients resembled the dissemination of VL caused by *L. infantum* seen in HIV–co-infected patients from the Mediterranean basin, which have symptoms of VL, such as splenomegaly and fever, in addition to skin lesions. Thus, on the basis of clinical history and signs and molecular typing of paired strains from the viscera and skin of the infected persons. In the Americas, mainly in Brazil, disseminated CL caused by *L. braziliensis*, *L. guyanensis*, and *L. amazonensis* is an emerging common health problem in non-immunocompromised persons. In severely immunosuppressed patients, such as those with HIV/AIDS, clinical manifestations of *Leishmania* infections can be atypical. Dermatotropic species can visceralize and viscerotropic species can disseminate from reticulo-endothelial sites to the skin and cause disseminated CL. In Ethiopia, three dermatotropic species can disseminate from reticulo-endothelial sites to the skin, all of our patients were diagnosed as cases of VL and disseminated CL.

In HIV/VL co-infected patients who have received ART and showed evidence of immune reconstitution, multiple lesions could also be clinically recognized as PKDL/VL associated with inflammatory syndrome (IRIS). However, until now, there are no clear clinical or laboratory criteria to define IRIS in leishmaniasis patients. Case-patient 1 had never received ART. Thus, the appearance of multiple cutaneous lesions with high parasite load is more likely to be related to a dissemination of hematogenous *L. donovani* parasites because of immune failure. Immunosuppressed patients are not able to mount an efficient T cell response for controlling *Leishmania* infections. Conversely, case-patient 2 had been receiving ART and was treated with SSG for VL but relapsed several times before the first onset of skin lesions. Clinical follow-up of this patient suggested the absence of immune reconstitution after initiation of ART. This finding and identification of high parasite load and genetically identical *L. donovani* in viscera and skin lesions led us to consider this patient as a case of VL with disseminated CL caused by severe immunosuppression (CD4 cell count < 200 cells/mm³).

Case-patient 3 could be considered as a case of VL and disseminated CL or PKDL/VL associated with IRIS. The main reasons for this diagnosis are a low CD4 cell count (< 50 cells/mm³) before initiation of ART, first appearance of skin lesions after six months of ART, and identification of genetically identical *L. donovani* parasites isolated from viscera and skin lesions. However, an increase of CD4 T cells from 40 to 93 cells/mm³ cannot signify an immune reconstitution or even an increase in lymphocyte effector function in this patient. Thus, this patient is most likely a case of VL with disseminated CL caused by severe immunosuppression (CD4 cell count < 200 cells/mm³).

Case-patient 4 could be considered as a case of VL and disseminated CL or PKDL/VL associated with IRIS. The main reasons for this diagnosis are severe immunosuppression (CD4 cell count < 50 cells/mm³) before initiation of ART, first appearance of skin lesions after six months of ART, and identification of high parasite load and genetically identical *L. donovani* in viscera and skin lesions. However, an increase of CD4 T cells from 40 to 93 cells/mm³ cannot signify an immune reconstitution or even an increase in lymphocyte effector function in this patient. Thus, this patient is most likely a case of VL with disseminated CL caused by severe immunosuppression (CD4 cell count < 200 cells/mm³).
lesions described in present and previous studies could be related to the good skin penetration of antimony.\textsuperscript{30}

In the Mediterranean basin, biochemical differences were observed between dermatotropic and viscerotropic isolates of \textit{L. infantum}.\textsuperscript{31} However, other studies in different Mediterranean countries\textsuperscript{32, 33} showed that this association was not absolute. Only some zymodemes were exclusively isolated from CL cases whereas others, including the predomninating zymodeme MON1, were found to cause VL and CL. A study in Sudan that used the cytochrome oxidase II gene for molecular characterization of \textit{Leishmania} isolates obtained from a case of mucosal leishmaniasis suggested that parasites invading the oral mucosa are distinct from parasites causing VL, perhaps representing a different subspecies of the \textit{L. donovani} complex.\textsuperscript{34} However, by using the most polymorphic markers, microsatellites, we did not observe any genetic differences in paired \textit{L. donovani} isolates from cutaneous lesions and viscera of the same HIV/AIDS patients.

Bayesian statistical analysis and distance-based analyses showed that genetic clustering of \textit{L. donovani} strains from Ethiopia does not correlate with disease phenotype. All strain pairs from the viscera and skin lesions and one strain from a patient with of PKDL/VL represented distinct MLMT types and were assigned to the NE/SD population previously identified, albeit to different sub-populations. This finding points to the absence of any correlation between disease phenotypes (disseminated CL with concomitant VL, PKDL/VL, VL, PKDL) and genotypes of \textit{L. donovani} from Ethiopia, and supports findings of studies in Sudan\textsuperscript{35,36} and the Indian subcontinent.\textsuperscript{37} This finding could suggest that host immunogenetic
background is important for predicting whether PKDL or disseminated CL will develop in *L. donovani*-infected persons.\(^{30,39}\)

In conclusion, our study shows that *L. donovani* is the causative agent of the disseminated CL cases described herein, and that strains isolated from disseminated skin lesions and viscera of the same patient during the VL episode are genetically identical. However, we cannot conclusively rule out the possibility that these cases are PKDL/VL.\(^{39}\) PKDL is thought to be uncommon in HIV/AIDS patients and so far only a few cases of PKDL in HIV-positive patients have been reported from other foci.\(^{40-42}\) However, in Ethiopia, PKDL is reported to be more common in HIV-positive patients than in HIV-negative VL patients.\(^{43}\) This finding is controversial but severity of immunosuppression in these patients is unknown because CD4 cell counts were not reported.

It is now increasingly believed that PKDL is associated with increased T cell responses to the parasites, which lead to inflammation.\(^{27-44}\) In our patients, we observed consistently low CD4 cell counts and high parasitic loads in splenic and bone marrow aspirates and skin lesions, which suggests that immunosuppression enables the parasites to multiply in the periphery. Therefore, we prefer to consider the cases described herein as cutaneous dissemination of visceral disease. Nevertheless, with the global increase of HIV infection in patients admitted to Gondar University Hospital, Ethiopia: the influence of antiretroviral treatment and other factors on outcome. *Clin Infect Dis* 46: 1702–1709.


