Liposomal Amphotericin B and rHuGM-CSF for treatment of visceral leishmaniasis in AIDS

Amfotericina B liposomiale e rHuGM-CSF nel trattamento della leishmaniosi viscerale in corso di AIDS

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■ **INTRODUCTION**

Human leishmaniasis is a parasitic disease caused by different species and sub-species of an early-branching unicellular eukaryote belonging to the order *Kinetoplastidae* and family *Leishmania,* subgenera *Leishmania* and *Viannia*. Humans are usually accidental hosts of these 2 mm long flies; most *Leishmania* species are considered to be parasites of wild animals, usually small mammals, a variety of rodents, or dogs; in a few cases domestic animals have also been implicated.

The disease is transmitted by insects as vectors, and there are about 200 species and subspecies of sandflies implicated in its transmission world-wide. Infection is geographically and ecologically widespread on all continents except Australia; the disease is highly endemic throughout northern Africa, the Middle East, parts of Europe, and Central and South America. The incidence of leishmaniasis is increasing, with many endemic areas reporting a 500% increase over the past seven years [1, 2].

The Leishmania parasite, a haemo-flagellate protozoan organism, is exclusively transmitted by the bite of a female sandfly of the genus *Phlebotomous* or *Lutzomyia*. Disease may range from self-limiting skin lesions to life-threatening visceral or muco-cutaneous forms.

Leishmaniasis consists of a cluster of diseases with diverse clinical manifestations, including cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis (Kala-azar). Intracellular survival of these parasites within human macrophages helps them to subvert the effector and regulatory functions of these cells. People with HIV infection are at higher risk of contracting the diseases if they live in or travel to endemic regions. It is currently estimated that 25-70% of adult visceral leishmaniasis cases are related to HIV, and 1.5-9% of AIDS cases suffer from newly acquired or re-activated visceral leishmaniasis [3]. In these co-infected patients, visceral leishmaniasis presents similar or more severe clinical symptoms than in immunocompetent patients and because of the former patients' immunological condition may exhibit additional specific characteristics, e.g., parasitization of unusual locations such as skin, digestive tract or lung.

For many decades, pentavalent antimonials constituted the standard treatment for visceral leishmaniasis. These agents have been used extensively and have been demonstrated to be efficacious and safe. During the last decade, however, the emergence of strains of microorganisms resistant to pentavalent antimonials has prompted the evaluation of alternative therapeutic agents, including pentamidine, interferon gamma, liposomal amphotericin B, and miltefosine. Major challenges in treating leishmaniasis include widespread resistance to pentavalent antimonial compounds, absence of safe chemotherapeutic agents, and treatment failure and relapses in HIV-leishmania co-infected patients.

In this report, we describe the safety and efficacy of combination treatment with liposomal amphotericin B and rHuGM-CSF immunotherapy used for the therapy of visceral leishmaniasis in a patient with AIDS.

CASE REPORT

A 37-year-old Italian male with a history of intravenous drug use, neurotoxoplasmosis and salmonellosis was admitted to our hospital in September 2001 for a 1-month history of high fever resistant to antibiotics and anti-febrile drugs. He was commenced on daily didanosine, stavudine, and nelfinavir in February 2000 when he was found to have a CD4+ count of 56 cells per mcl and a viral load of 46,400 copies/mL. However, a few months later he decided not to take antiretroviral drugs.

In April 2001 he had been admitted for neurotoxoplasmosis, which responded to a course of pyrimetamine and clyndamycin. During the next two months a chronic febrile diarrhoea appeared, and stool and blood examinations disclosed Salmonella B infection treated with ciprofloxacin. The patient responded favourably to treatment and defervesced.

In June 2001 he commenced antiretroviral treatment which included zidovudine, lamivudine, and abacavir. At that time the CD4+ cell count was 61 per mcl, while a plasma HIV RNA load was 105,000 copies per mL. However, one month later the patient again became febrile, and a marked hepatosplenomegaly was evident on an ultrasonogram of the abdomen. On physical examination he was febrile (temperature, 38-39°C). He was thin with a 5 cm enlarged spleen and a palpable liver.

Laboratory investigations showed a WBC count of 1.20×10^3/mmc (0.77×10^3/mmc neutrophils, 0.30×10^3/mmc lymphocytes), a platelet count of 51,000/mmc, a haemoglobin value of 7.8 g/dL, a haematocrit value of 23%, an ESR of 98 mm/h, a C-reactive protein of 6.3 mg/dl, an LDH value of 828 U/L, a total protein value of 5.51 g/dL, serum IgG 740 mg/dL, IgA 71, and IgM 23 mg/dL. The CD4+ cell count was 31 per mcl, while a plasma HIV RNA load was undetectable below 50 copies per mL.

A chest radiograph was negative; a second ultrasonogram confirmed a marked hepatosplenomegaly. Numerous blood and urine cultures for bacterial, fungal and viral pathogens, Mantoux test, cryptococcal antigen, toxoplasma, and cytomegalovirus antibody were negative. Bone-marrow examination for microscopy and cultures for acid-fast bacilli were negative. Histopathologic evaluation of bone marrow biopsy specimens revealed the presence of amastigote leishmania forms, and leishmania serology was positive, 1:256.

Empirical treatment with antibiotics was given but discontinued after blood cultures were found to be negative, the fever persisted and bone marrow examination showed leishmaniasis. Visceral leishmaniasis was defined as identification of the amastigote form of leishmania in bone marrow, positive serological test with immunofluorescence antibody test in a patient with manifestations compatible with visceral leishmaniasis, including fever (defined as temperature >38°C), hepatosplenomegaly, anemia (defined as a haemoglobin value <9 g/dl), leukopenia (defined as <4000 WBCs/mL of blood), and thrombocytopenia (defined as <150,000 platelet/mL).

Liposomal amphotericin B was given in a dose of 4 mg/kg per day for five consecutive days, and subsequently on day 10, 17, 14, 31 and 38. The patient also received rHuGM-CSF, 150 mcg subcutaneously twice weekly for 12 consecutive weeks, without adverse events and a dramatic clinical improvement and a reduction of splenic size; after 24 months he remained well on HAART, with stavudine, lamivudine, and abacavir.

DISCUSSION

Several observations have emphasized the increasing importance of visceral leishmaniasis as an opportunistic infection among HIV-positive patients in areas where both infections are endemic. People with HIV infection are at increased risk of developing leishmaniasis if they live or travel to endemic regions. Co-infected patients may serve as carriers of Leishmania in the blood, a potential source of infection for the sandfly. Needle sharing by intravenous drug users and unprotected male homosexual intercourse have also been indicated as predisposing conditions for contracting leishmaniasis in these patients.

The majority of HIV-positive patients with visceral leishmaniasis present fever, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia and CD4+ lymphocyte count $<200 \times 10^6$/l. However, unusual features in immunosuppressed patients co-infected with HIV may include absence of fever and visceromegaly, involvement of the pericardium, liver, gastrointestinal mucosa and dissemination to the skin[4-7]. The clinical manifestations of visceral leishmaniasis in HIV-infected patients may be influenced by CD4+ count; pa-

tients with CD4+ count <50/mcl have a lower frequency of the clinical triad of fever, splenomegaly, and hepatomegaly. In these patients findings of *Leishmania* amastigotes in atypical locations may be frequent, and involvement of the gastrointestinal tract or the respiratory tract is not uncommon [8]. A case of vasculitis with digital necrosis caused by *Leishmania* species has also been described in a woman infected with HIV [9].

Leishmania/HIV co-infection has emerged as a result of the increasing overlap between leishmaniasis, mainly visceral and more rarely cutaneous, and AIDS, which is due to the spread of AIDS pandemic to rural area and that of visceral leishmaniasis to suburban areas. The spread of HIV infection in leishmaniasis-endemic countries has resulted in a sharp increase in the reported numbers of cases of HIV-*Leishmania* co-infection in recent years. Cases of co-infection have so far been reported from 33 countries around the world, most of the cases being recorded in south-western Europe [10]. To date, 1,627 cases have been notified from Spain, France, Italy and Portugal [10]. While *Leishmania*/HIV co-infection is increasing in eastern Africa, cases of co-infection are expected to diminish in south-western Europe due to the new highly active antiretroviral therapy (HAART).

In 1998, a worldwide WHO/UNAIDS surveillance network was established, which now includes 28 member institutions. In south-western Europe, the surveillance system based on institutions is now well established [10].

With the spread of HIV, visceral leishmaniasis has become more prevalent, and unusual presentation often occurs. Reactivation of asymptomatic or previously "healed" *Leishmania* infections is common with the onset of AIDS. Leishmania species that normally cause only cutaneous disease may be present with visceral leishmaniasis. In addition, co-infections of *Leishmania* and HIV are often resistant to treatment and substantially accelerate the progress of AIDS [11].

Most cases of HIV-related Kala-azar have so far been reported in southern Europe along the Mediterranean basin. In the Mediterranean basin, *Leishmania infantum* is the infectious agent of both visceral and cutaneous leishmaniasis and has been shown to be an important opportunistic parasite in patients with AIDS. Indeed, up to 9% of these patients may suffer from newly acquired or reactivated visceral leishmaniasis [1-2].

The majority of leishmaniasis cases occur in patients in the advanced stages of HIV disease, and the depletion of CD4+ cells induced by HIV can alter any clinical aspect of Kala-azar, including the response to treatment and propensity for relapse [12]. The HIV viral load is significantly higher in *Leishmania* co-infected patients and this may be partly due to Th2 immune activation, as demonstrated by higher plasma levels of IL-4, -6 and –10, suggesting that *Leishmania* infection alters HIV-specific immunity [13].

Successful host defence in experimental visceral leishmaniasis involves a complex Th1 cell-dependent, multicytokine-mediated mechanism, probably initiated by interleukin-12 and then driven primarily by interferon-γ, and culminating in macrophage activation, intracellular killing within tissue granulomas, and long-term quiescence in residual surviving parasites. This mechanism may explain why the majority of human infections caused by visceralizing strains of *Leishmania* remain sub-clinical and do not require therapy, while in those patients who do progress to fully established visceral disease, presumably this protective Th1 cell response failed to develop or was deactivated.

In immunocompetent individuals, visceral leishmaniasis is associated with a clear humoral response, due to polyclonal B-cell activation, and a suppression of the cellular response to the parasite which is restored after successful chemotherapy. The T-cell-mediated immune response is critical for cure and protection in secondary infections. Immune responses are modified in *Leishmania*/HIV co-infected patients, and immunologic abnormalities caused by HIV infection may lead to *Leishmania* dissemination with atypical locations, a clear tendency to relapsing disease and poor response to treatment.

Specific *Leishmania* antibodies are often not detectable in the blood, probably as consequence of an oligoclonal B-cell response due to the absence of T-cells that can recognize *Leishmania* antigens and stimulate the B cells [12]. Compared to immunocompetent patients with visceral leishmaniasis, co-infected patients present lower levels of specific antibodies in spite of treatment [3].

During HIV infection there is evidence of a selective depletion of CD4+ lymphocyte cells, a severe impairment in the production of Th1 cytokines with prevalence of a Th2 cytokine profile, and a certain degree of immunological and

functional abnormalities of macrophages associated with a defect of the inductive signal required for the activation of macrophages and macrophage dysfunction. Such immunological dysfunctions may result in the loss of control of intracellular multiplication and reactivation of *Leishmania* in latently infected macrophages in HIV-positive individuals.

The inability of antibody response to control the parasite and the absence of specific T-cell immunity to *Leishmania* would explain the high frequency of relapses reported in these subjects [3]. It has been suggested that both HIV and Leishmania may affect gene expression of one another, and thus following infection with HIV, individuals with pre-existing and asymptomatic *Leishmania* infection may develop disseminated disease or may be susceptible to developing new infections [14].

In a recent study, an association between an up-regulation of three beta chemokines (MIP-1aalpha, MIP-1beta, and RANTES) and a chemokine receptor (CCR5), in relation to *Leishmania* and HIV co-infection has been reported [15]. Other immunologists have suggested the clinical development of oligodeoxynucleotide containing CpG motifs as immunoprotective agents against *Leishmania* in normal and HIV-infected patients [16].

Definitive diagnosis requires tissue specimens, which are obtained by organ needle aspiration, and rests on microscopic demonstration of characteristic amastigote forms in stained smears. The test for serum antibody to *Leishmania donovani* is positive in only 34% of these cases in HIV-infected patients vs 95% of cases in immunocompetent patients [17]. Bone marrow, the spleen and the lymph nodes are the tissues most often sampled in patients with suspected infection. The diagnostic sensitivity of splenic aspiration is high (95%-98%), but the procedure carries a risk of bleeding; the sensitivity of examination of bone marrow specimens is considered to be lower (53%-95%). Culture or PCR testing of aspirate material improves parasitological yield, but these methods are seldom undertaken outside research laboratories. The results of a recent study have suggested the utility of a PCR-RFLP analysis in detecting leishmaniasis re-infection in HIV-positive patients [18].

A range of assays have been developed to detect anti-leishmanial antibodies as well as parasite DNA in peripheral blood. However, the usefulness of these assays is limited by the variable sensitivity and specificity, requirements for equipped laboratory and costs. Recently, a rapid, accurate, and non-invasive method of diagnosis of Kala-azar was developed to detect circulating anti-leishmanial antibody anti-K39 IgG by use of laboratory ELISA testing. K39 is an epitope which appears conserved on amastigotes of Leishmania species that cause visceral infection, and circulating anti K39 IgG is detectable in 95-100% of patients who have Kala-azar.

This strip test detection of anti-K39 IgG appears a clinically promising diagnostic guide in those with suspected kala-azar [19]. Use of this specific K39 antigen may also improve antibody detection in immunosuppressed patients.

Despite the associated adverse reactions and the need for prolonged parenteral treatment, pentavalent antimony has remained the traditional, first-line therapy for Kala-azar throughout the world, primarily because it is affordable and effective and is surely a time-tested therapy. However, large-scale therapeutic failure of this conventional treatment has emerged in recent years, suggesting steady erosion in the capacity of pentavalent antimony to induce long-term cure in patients with Kala-azar in several major epidemic area of Kala-azar [20, 21]. The cure rate after 1 drug course is about 95%, but in adult individuals adverse reactions such as pancreatitis and/or cardiac abnormalities are common.

By means of active surveillance methods, antimony-associated mortality was found to be as high as 7% in adult HIV-negative patients with or without underlying disease in Sicily [23]. Treatment of visceral leishmaniasis with pentavalent antimony resulted in death rates of 4.8% to 20% in other surveys. Relapse rates of visceral leishmaniasis after sodium stibogluconate therapy varied between 1 and 18% in several studies. A number of factors, primarily thought to be related to years of providing incomplete treatment and suboptimal dosing that led to drug resistance among parasites, have been implicated in the occurrence of complications after treatment and have contributed to the failure of sodium stibogluconate for the treatment of Kala-azar [22].

In conclusion, conventional antimony treatment for visceral leishmaniasis has several main drawbacks, including the toxicity of pentavalent antimonials, the prolonged therapy required to achieve cure, and the risk of relapse even after initial good response. Pentamidine

has been used extensively for the treatment of refractory visceral leishmaniasis, but due to the decline in efficacy, serious toxicity causing diabetes mellitus and high cost, its use has declined. However, it has been used effectively in the treatment of cutaneous and muco-cutaneous leishmaniasis [24]. Paromomycin, alone or in combination with sodium stibogluconate, has been shown to be highly efficacious and well tolerated for the treatment of visceral leishmaniasis. Ototoxicity and renal toxicity are the major historical toxicities of paromomycin [24]. Several studies have suggested the efficacy of Interferon-γ (IFN-γ) therapy in patients with visceral leishmaniasis; in addition, it has been demonstrated that combined therapy with antimony plus IFN-γ may accelerate parasitologic response and apparent cure in patients with Kala-azar, with the initial efficacy largely maintained at 6 months [25].

Miltefosine, a phosphocholine analogue which interferes with cell signalling pathways in tumours, has been shown to be highly active against leishmania in vitro and in animal models. This oral drug was tried against human visceral leishmaniasis at a dose of 100 mg per day for 4 weeks and achieved 97% cure in a phase-2 trial. Mild to moderate gastrointestinal side effects were observed in 62% of patients. Oral miltefosine was effective at the first treatment course in an HIV-infected patient with a visceral leishmaniasis resistant to sodium antimony gluconate, with a clinical and parasitological cure at the end of treatment [26].

Amphotericin B is known to be effective in the treatment of visceral leishmaniasis but because of its supposed toxicity it has so far been used only as a second-line treatment. In recent years, the search for alternative treatments has led to recognition of lipid-associated amphotericin B formulations as powerful anti-leishmanial agents. Lipid formulations of amphotericin B have demonstrated reduced toxicity compared with conventional amphotericin B desoxycholate. Three preparations with different pharmacokinetic properties are currently approved: amphotericin B lipid complex (Abelcet®), liposomal amphotericin B (AmBisome®) and amphotericin B colloidal dispersion (Amphocil®).

Of these three lipid formulations, liposomal amphotericin B (AmBisome®) has the best safety profile. Liposomal amphotericin B was shown to be effective and non-toxic for visceral leishmaniasis treatment in immunocompetent individuals in the Mediterranean area. The reg-imen for liposomal amphotericin B approved by the US Federal Drugs Administration for visceral leishmaniasis consists of 3 mg/kg given on days 1-5, 14, and 21 (total dose, 21 mg/kg). However, commercially available lipid formulations of amphotericin B are expensive, and the cost of even short course or low dose regimens represents an insurmountable obstacle in the developing world.

HAART does not appear to induce evolution of latent leishmaniasis into symptomatic disease, since it leads to increasing T cell counts with a reduced immune system activation, and to increased levels of T-helper type 1 cytokines that are associated with control of leishmaniasis [27]. French authors observed a significant decline in the incidence of visceral leishmaniasis in HIV-infected individuals after 1996, associated with the introduction of HAART, which resulted in a 59% decreased risk in people treated with antiretroviral therapies [28].

In some cases, association of liposomal amphotericin B therapy combined with potent antiretroviral treatment has been associated with long-term remission of HIV-associated leishmaniasis [29]. Th1 cytokines have been found to restore cytotoxic macrophage functions that are essential for the control of intracellular growth of *Leishmania*. They underscore the clinical potential of these cytokines as therapeutic adjuvants for the treatment of leishmaniasis in patients co-infected with HIV [14].

The haematopoietic growth factor GM-CSF has well-documented stimulatory effects on phagocytic and metabolic functions of monocytes and macrophage, including increased synthesis of molecules toxic to microbes and the release of other pro-inflammatory cytokines resulting in inhibition and/or killing of *Leishmania* and other intracellular pathogens [30]. It is well known that GM-CSF can inhibit the intracellular replication of bacteria such as *Salmonella*, *Listeria*, and *Mycobacteria*, or protozoa such as *Leishmania*, which rely on the intracellular microenvironment for their proliferation and utilize macrophages in tissue as a part of their life cycle [30-31]. IL-6, a cytokine synthesized by T cells and macrophages and recognized for its pro-inflammatory properties and pleiotropic effects on diverse cell types, might modulate the cytokine-enhanced anti-leishmania activity in human macrophages [33].

GM-CSF induces effects potentially beneficial in visceral leishmaniasis, such as blood monocyte mobilization, macrophage activation, and

amelioration of granulocytopenia. Several experimental observations have suggested that GM-CSF can be used as an anti-leishmanial treatment [34, 35]. The efficacy of GM-CSF in combination with pentavalent antimony was investigated in 20 neutropenic patients with acute leishmaniasis. All patients had complete resolution of disease symptoms at three months; GM-CSF given subcutaneously at a dose of 5 mcg/kg daily for ten days was well tolerated, reversed neutropenia rapidly and reduced the number of secondary infections in patients with leishmaniasis [35].

We conclude that treatment with rHuGM-CSF partially restored anti-leishmanial immune responses in our patient, and that the development of novel immunotherapeutic protocols using combinations of rHuGM-CSF and liposomal amphotericin B may improve clinical response in HIV-infected patients with leishmaniasis. In addition, it is possible to treat affected patients with outpatient administration of liposomal amphotericin B and rHuGM-CSF, making them more feasible options for therapy.

SUMMARY

In recent years, several reports have emphasized the increasing importance of visceral leishmaniasis as an opportunistic infection among HIV-positive patients in areas where both infections are endemic. Major challenges in the treatment of leishmaniasis include widespread resistance to pentavalent antimonial compounds, absence of safe chemotherapeutic agents, and treatment failure and relapses in HIV-leishmania coinfected patients. Despite the associated adverse reactions and the need for prolonged parenteral treatment, for several decades pentavalent antimony has remained the traditional, first-line therapy for kala-azar throughout the world. However conventional antimony treatment for visceral leishmaniasis has several main drawbacks, including the toxicity of pentavalent antimonials, the prolonged therapy required to achieve cure, and the risk of relapse even after initial good response.

In recent years, the search for alternative treatment has led to the recognition of lipid associated amphotericin B formulations as powerful antileishmanial agents. Several observations have also suggested that GM-CSF can be used as an antileishmanial treatment. Indeed, GM-CSF induces potentially beneficial effects in visceral leishmaniasis, such as blood monocyte mobilization, macrophage activation, and amelioration of granulocytopenia. In this report, we describe the safety and efficacy of combination treatment with liposomal amphotericin B and rHuGM-CSF immunotherapy used for the therapy of visceral leishmaniasis in a patient with AIDS.

RIASSUNTO

Negli ultimi anni numerose segnalazioni della letteratura hanno evidenziato la crescente importanza della leishmaniosi viscerale quale infezione opportunistica nei pazienti HIV-positivi nelle aree geografiche laddove entrambe queste infezioni hanno carattere di endemicità. Le maggiori sfide poste dalla terapia della leishmaniosi sono da ricercare nella resistenza ampiamente diffusa ai composti antimoniali pentavalenti, nell'assenza di agenti chemioterapici sicuri, e infine nei fallimenti e nelle recidive che si verificano in quei pazienti contemporaneamente affetti da leishmaniosi e HIV.

A dispetto delle reazioni avverse e della necessità di una terapia parenterale prolungata, l'antimonio pentavalente ha costituito, per svariati decenni e in tutto il mondo, la terapia tradizionale, di prima scelta, per il trattamento del kala-azar. Tuttavia il trattamento convenzionale con antimonio presenta aspetti negativi non trascurabili, inclusi la tossicità degli antimoniali pentavalenti, il prolungato periodo di terapia necessario per giungere a guarigione, e il rischio di ricaduta anche a seguito di un'iniziale risposta soddisfacente. Più di recente, la ricerca di trattamenti alternativi ha condotto alla formulazione dell'amfotericina B liposomiale quale potente agente anti-leishmania. Numerose osservazioni hanno inoltre suggerito

che GM-CSF può essere impiegato nella terapia anti-leishmania; esso induce un effetto potenzialmente benefico nella leishmaniosi viscerale, caratterizzato, ad esempio, dalla mobilizzazione dei monociti, dall'attivazione macrofagica, e dal miglioramento della granulocitopenia.

Nel presente articolo, gli autori descrivono la sicurezza e l'efficacia del trattamento di associazione con amfotericina b liposomiale e immunoterapia con rHuGM-CSF adottato per la terapia della leismaniosi viscerale in un paziente con AIDS.

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