

Visceral Leishmaniasis in a German Child Who Had Never Entered a Known Endemic Area: Case Report and Review of the Literature

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We describe a case of visceral leishmaniasis in a 15-month-old German child. Diagnosis was significantly delayed because the patient had no history of travel to known endemic areas. Congenital or blood transfusion-associated leishmaniasis was ruled out. Possible modes of transmission (including a potential new autochthonous focus of the disease in central Europe) are discussed.

Leishmania organisms are flagellated protozoa that are usually transmitted to mammalian hosts by sandflies. In Europe, cutaneous and visceral leishmaniasis are endemic in all Mediterranean countries. *Leishmania infantum* (dermotropic as well as viscerotropic strains) accounts for most of the clinical cases [1]. In the classical foci of southern France, infected dogs are an important natural reservoir of *L. infantum* [2]. The dogs infect sandflies even if the dogs appear clinically asymptomatic [3]. So far, truly autochthonous human cases of leishmaniasis have not been reported from central European countries such as Germany, Switzerland, Poland, Belgium, and The Netherlands. We describe an unusual case of visceral leishmaniasis caused by *L. infantum* in a 15-month-old German child who had never

stayed in a known area of endemicity and in whom other routes of infection (e.g., congenital leishmaniasis or blood transfusion) were excluded.

Case report. In September 1997, a 15-month-old German boy from a suburb of the city of Aachen, Germany, became ill with recurrent upper airway infections that were not controlled by oral antibiotic and mucolytic therapy. The boy had never left Germany, other than to take a weekend trip to Schouwen (an island west of Rotterdam, The Netherlands) in August 1996 and to stay in Burgh (located on the island of Schouwen) for 1 week in April 1997. The family had no pets. In October 1997, the child was admitted to the pediatric department of a regional hospital for anemia and inappetence. Because of persistent fever, splenomegaly, and moderate pancytopenia, the child was admitted to the pediatric hospital at the University of Aachen to exclude the possibility of a systemic malignancy.

At the time of admission, the 16-month-old boy (weight, 11.2 kg; height, 78 cm) had anemia, a temperature of 39.2°C, and a few bilateral cervical lymph nodes with a diameter of ≤ 0.5 cm. The liver and spleen were palpable 2 cm and 8 cm below the right costal margin and the left costal margin, respectively. Laboratory studies gave the following values: hemoglobin, 7.4 g/dL; hematocrit, 22%; erythrocyte count, 3.4×10^6 cells/mm³ (9.3% reticulocytes); WBC count, 4800 cells/mm³ (13% neutrophils, 11% band forms, 69% lymphocytes, 5% monocytes, and 1% eosinophils); platelet count, 97,000 cells/mm³; erythrocyte sedimentation rate, 14 mm in the first hour and 93 mm in the second hour; C-reactive protein, 2 mg/dL; and lactate dehydrogenase, 280 U/L. A bone marrow aspirate showed an intact and stimulated erythro-, granulo-, and thrombopoiesis and no parasites. There were no signs of malignancy, and the child was discharged. His condition was thought to reflect a prior viral infection with a reactive bone marrow.

Four weeks later, the child was readmitted to the pediatric university hospital with persistent fever (temperature, $\leq 40^\circ\text{C}$) that remained refractory to various oral and iv antibiotic therapies. In the meantime, the child had received a transfusion of packed erythrocytes, and from the middle of November onward, he received granulocyte colony-stimulating factor (G-CSF) 3 times per week. At the time of admission, the liver and spleen were found to be palpable 4 cm and 8 cm below the right costal arch and the left costal arch, respectively.

Laboratory studies obtained the following values: hemoglobin, 6.1 g/dL; hematocrit, 18%; WBC count, 5100 cells/mm³ (29% neutrophils, 6% band forms, and 61% lymphocytes with

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toxic granulations); platelet count, 37,000 cells/mm³; and C-reactive protein, 4.2 mg/dL. Bone marrow smears and punch cylinders showed that differentiated cells of all hematopoietic lineages were sufficiently present. The erythropoiesis:granulopoiesis ratio was 1:1.5, and in both lineages, an increased number of immature cells was observed, compatible with a myelodysplastic syndrome (type refractory anemia). Immunophenotyping of the bone marrow provided no evidence that the patient had a systemic malignant disease. The intracutaneous reaction to ubiquitous recall-antigens was significantly decreased. Serum ferritin (979 µg/L), IgM (7.87 g/L), and IgG (4.38 g/L) levels were increased.

Because the fever was refractory to broad-spectrum antibiotic therapy, oral treatment with prednisolone (2 mg/kg/day) was started in December 1997. This led to defervescence and clinical improvement that was significant enough for the child to be discharged while taking prednisolone (1 mg/kg/day) and G-CSF (3 times per week). Soon thereafter, the daily bouts of intermittent fever recurred, which, however, only slightly impaired the child.

On the basis of the clinical course and the laboratory parameters, we suspected a case of hemophagocytic lymphohistiocytosis. This was further supported by the following findings: (1) the increased percentage of activated macrophages (20.2% were CD80 positive, and 41.4% were CD86 positive), the expression of B7 (CD80) costimulatory molecules on T lymphocytes, and an almost complete lack of natural killer cells in the peripheral blood; and (2) the strongly increased concentrations of soluble IL-2 receptor α chain (20,756 pg/mL) and of proinflammatory cytokines (TNF- α , 321.2 pg/mL; IL-1 β , 44.8 pg/mL; IL-6, 69.6 pg/mL; and IL-8, 78.4 pg/mL) in the serum of the patient.

In January 1998, the child was again readmitted to the same hospital with persistent clinical signs (fever; hepatosplenomegaly; hemoglobin, 8.4 g/dL; WBC count, 3600 cells/mm³; and platelet count, 9000 cells/mm³). Analysis of another bone marrow aspirate revealed a striking increase in the number of macrophages (histiocytes). Numerous macrophages contained vacuoles with suspected protozoan parasites. Giemsa staining and anti-*Leishmania* immunofluorescence assay demonstrated numerous intra- and extracellular *Leishmania* amastigotes in bone marrow smears. *Leishmania* kinetoplast DNA was detected in peripheral blood; peripheral blood yielded *Leishmania* parasites within 2 days of culture. The isolated parasites were typed as *L. infantum* (World Health Organization code for the strain, MHOM/00/98/LUB1). The serum sample from the child contained a high titer (>1:3200) of anti-*Leishmania* antibodies. Visceral leishmaniasis with secondary hemophagocytic lymphohistiocytosis was diagnosed. The patient was treated with amphotericin B (AmBisome; 3.5 mg/kg/day) for 10 days and rapidly recovered. We followed established protocols for ad-

ministration of amphotericin B [4, 5]. Within 3 days of treatment, the child's temperature fell to $\leq 38^{\circ}\text{C}$, and his condition improved dramatically. Four days after therapy was concluded, the boy returned home. Control bone marrow aspirates obtained in March and September 1998 were found to be negative for *Leishmania* parasites by means of microscopy, culture, and PCR. In March 1998, the spleen was still enlarged (5 cm below the costal margin). In May 1998, the hemoglobin level was 10.5 g/dL, the WBC count was 6100 cells/mm³, and the platelet count was 149,000 cells/mm³. One year after treatment, the child was in an excellent general condition, and the blood values (hemoglobin, 13.2 g/dL; WBC count, 6300 cells/mm³; and platelet count, 178,000 cells/mm³) and the size of the liver and spleen were normal.

Materials and methods. Serial 2-fold dilutions of bone marrow aspirates and peripheral whole blood samples obtained from the patient and his mother were cultured in modified Schneider's *Drosophila* medium [6] at 28°C, 5% CO₂, and 95% humidity in air for a total of 21 days. DNA was isolated from bone marrow aspirates or peripheral blood samples with the use of the Dynabead DNA Direct System II (Dyna). Extracted DNA was analyzed for the presence of a 120-bp fragment of *Leishmania* minicircle kinetoplast DNA by means of PCR [7].

Air-dried bone marrow smears were prepared as follows: they were fixed with acetone; blocked with phosphate-buffered saline/1% bovine serum albumin and 1% saponin; incubated sequentially with rabbit anti-*Leishmania major* IgG [8] that cross-reacts with *Leishmania donovani* and *L. infantum*, and with affinity-purified dichlorotriazinylamino-fluorescein-conjugated donkey anti-rabbit IgG F(ab')₂ fragments (Dianova); and mounted with Mowiol (Hoechst) containing 1,4-diazabicyclo-2,2,2-octane before analysis with a fluorescence microscope (Axiophot; Zeiss).

Isoenzyme analysis with 15 different enzymes was performed using starch gel electrophoresis [9] or multilocus enzyme electrophoresis on cellulose acetate plates [10]. The reference strain for the isoenzyme analysis on starch gels was MHOM/FR/78/LEM75 (zymodeme MON-1). For the multilocus enzyme electrophoresis, 11 different stocks of *L. infantum* (originating from different countries of the Mediterranean basin), 2 stocks of *Leishmania chagasi*, and 1 stock each of *L. donovani*, *L. major*, and *Leishmania tropica* were used.

Random amplified polymorphic DNA (RAPD) analysis was performed using 16 different primers that were used independently for the amplification of genomic DNA isolated from the patient's *Leishmania* strain as well as from a number of reference strains [11]. The whole internal transcribed spacer region, which is located between the small- and large-subunit RNA gene and which includes the 5.8S RNA gene, was amplified using primers LITSV (5'-ACA CTC AGG TCT GTA AAC-3') and LITSR (5'-CTG GAT CAT TTT CCG ATG-3'), which spe-

cifically amplify internally transcribed spacer sequences of *Leishmania* species. The PCR reaction mixture (50 μ L) contained 10 ng template DNA; 25 pmol each primer; 10 mM Tris/HCl, pH 8.0; 50 mM KCl; 1.5 mM MgCl₂; 200 μ M each dNTP; and 2 U Taq polymerase. The PCR conditions were as follows: 5 min at 95°C; 35 cycles of 20 s at 95°C, of 30 s at 50°C, and of 1 min at 72°C; and a final extension of 6 min at 72°C. The PCR products were digested with the use of restriction enzymes *Hae*II or *Cfo*I. Restriction fragments were separated on a 1.3% agarose gel.

The core sequence of phage M13 (5'-GAG GGT GGC GGT TCT-3'), the simple repeat sequence (GACA)₄ (5'-GAC AGA CAG ACA GAC A-3'), and the T3B oligonucleotide (5'-AGG TCG CGG GTT CGA ATC C-3'), which was derived from intergenic spacers of tRNA genes, were used as single primers in the PCR fingerprinting assays [12].

Discussion. The *Leishmania* strain isolated from the child was typed as *L. infantum* in 3 different laboratories that used either biochemical or molecular techniques. Isoenzyme analysis on starch gels revealed that the strain isolated from the child was identical to the reference strain *L. infantum* MON-1, which is the zymodeme most frequently isolated from patients in the Mediterranean basin who have visceral leishmaniasis. This was confirmed by means of multilocus enzyme electrophoresis, which showed that the patient strain clustered with parasite stocks from various countries, including France, Tunisia, and Morocco. Therefore, the geographical origin of the patient strain remains unknown. Molecular analysis of the patient strain—done either by (1) restriction enzyme analysis of the PCR-amplified internally transcribed spacer region, followed by PCR fingerprinting with 3 different primers, or (2) random amplified polymorphic DNA analysis with 16 different primers—unambiguously confirmed attribution of the isolate to the species *L. infantum*. Again, the results of these analyses did not allow us to draw any conclusion regarding its geographical origin.

This case of visceral leishmaniasis demonstrates that a medical history negative for traveling to known areas of endemicity is insufficient to exclude visceral leishmaniasis as a possible diagnosis in a patient with fever, hepatosplenomegaly, and pancytopenia. It also reminds us that the microscopical analysis of bone marrow smears has a limited sensitivity to diagnose visceral leishmaniasis during the early phase of infection. The first 2 bone marrow aspirates were negative for intracellular parasites in our patient. This case also illustrates that culture of a peripheral blood sample obtained from a patient with severe kala-azar can yield *L. infantum*, even if there was no underlying primary immunodeficiency [13]. This report raises important questions about the epidemiology and possible new modes of transmission of visceral leishmaniasis in central Europe. Several routes of infection are feasible and deserve to be discussed.

While the mother of our patient was pregnant with him (weeks of gestation, 20–22), she spent a 2-week vacation on Lanzarote, which is one of the Canary Islands. Although this region is not known to be endemic for visceral leishmaniasis, late-onset congenital leishmaniasis was nevertheless considered as a diagnosis and was excluded. Cases of congenital leishmaniasis are rare and have usually been associated with mothers who had contracted severe kala-azar before or during pregnancy [14]. One report [15] suggested that *L. infantum* was transmitted from a subclinically infected mother to her child. However, this mother, who had a bout of diarrhea that lasted for 4 weeks in the second trimester of pregnancy and who had premature contractions that occurred from the 28th week of pregnancy onward, had visited several areas of endemicity before her pregnancy, and was found to be seropositive for anti-*Leishmania* antibodies [15]. The mother of our patient, in contrast, had never developed any severe illness or clinical signs of visceral leishmaniasis during or after her pregnancy. In addition, her serum sample tested negative for antibodies against *Leishmania*. Finally, no *Leishmania* parasites were detected in a bone marrow aspirate by means of Giemsa staining, culture in modified Schneider's medium, and PCR analysis, thereby further excluding the hypothetical possibility of a clinically asymptomatic, persistent infection.

Acquisition of visceral leishmaniasis as a result of the transfusion of blood has been documented [16, 17]. However, our patient had never received any transfusions or injections of blood or blood products before the onset of his illness.

In areas of nonendemicity, malaria infections due to the import of *Plasmodium* species—infected *Anopheles* mosquitoes via aircraft or the baggage of tourists have been repeatedly suspected [18–21]. *Leishmania* parasites, however, are transmitted by sandflies, which are much more fragile than *Anopheles* species and are much less tolerant to temperature changes. Leishmaniasis acquired as a result of the import of *Leishmania* parasites/sandflies via baggage or an airport appears to be unlikely. However, the transfer of infected sandflies over shorter distances and under less harsh conditions (e.g., inside camping vehicles), is possible. In this context, it is important to note that, in June 1997 (2 months before the onset of illness), our patient and his parents spent a 3-week vacation in Füssen, a small town in southern Germany, where they stayed at a campsite frequently visited by tourists returning from Mediterranean countries.

Cases of visceral leishmaniasis in dogs and infants have been described outside the classical Mediterranean foci but are poorly appreciated in the current literature. Many of the primary reports are not listed in the MEDLINE database. In 1931, visceral leishmaniasis was diagnosed in a 5-year-old child who had never left the Vosges in northeastern France (close to the border of Germany) [22]. Later, autochthonous cases of canine

and/or human visceral leishmaniasis were reported in central France (e.g., in the vicinity of Paris) as well as in several departments of northern France at the shore of the English Channel and close to the border of Belgium (reviewed in Guilhon [23]). Dogs that were originally infected with *L. infantum* in southern France (e.g., during a vacation stay by their owners) and that were then moved back to northern provinces are thought to form the reservoir for the subsequent transmission of the *Leishmania* species to other dogs and infants via the sandfly species *Phlebotomus perniciosus* [23].

There are a few reports of visceral leishmaniasis in dogs from The Netherlands, Switzerland, or Germany that had never been abroad or been in contact with infected dogs and whose mothers had also never stayed in an area of endemicity [24–27]. The 2 German dogs with kala-azar were from the area of Cologne (~60 km east of the hometown of our patient) and from Landsberg/Lech (~55 km north of Füssen) [26].

A possible case of autochthonous human cutaneous *Leishmania* infection (caused by *L. infantum* but incorrectly attributed to *L. tropica* by the authors) was observed in a 20-year-old Swiss patient who had never left southern Switzerland (other than to make a brief visit to Spain 30 months before he developed the skin lesion) [28]. *P. perniciosus* is prevalent in Switzerland, and experimentally infected *P. perniciosus* females were capable of transmitting *L. infantum* to Syrian golden hamsters [29]. In Austria, 2 cases of autochthonous kala-azar have been suspected during the past 40 years [30, 31]. The first patient was a 36-year-old woman from lower Austria who had never been abroad [30]. Visceral leishmaniasis was diagnosed on the basis of clinical signs and a positive bone marrow smear but was not confirmed by serological tests or by culture of the parasites. The patient improved clinically without specific therapy. The second patient was the 3-year-old daughter of a Nigerian father and a Ghanaian mother; her parents had lived in Austria for 15 years and had never visited a known area of endemicity during that time [31]. This patient had parasites in the bone marrow and antibodies against *Leishmania* species in her serum sample; she was successfully treated with sodium stibogluconate followed by amphotericin B.

On the basis of these sporadic reports of canine and human visceral leishmaniasis in various central European countries, it is possible that our patient was infected while he was staying in The Netherlands, in Füssen (southern Germany), or in his hometown near Aachen (which is close to Belgium and The Netherlands and is only 150–250 km away from several areas of France in which autochthonous cases of human visceral leishmaniasis have been described [23]). Infection could have occurred via direct contact with infected dogs, which frequently develop skin lesions packed with infective *Leishmania* and which excrete the parasites with the urine [32–34] or via sandflies. It is important to note that, recently, the first sandfly species (*Phlebotomus mas-*

cittii Grassi, 1908) was found in Germany at 3 different locations along the upper Rhine valley between Freiburg and Basel, close to the border of France and Switzerland [35]. However, it remains to be demonstrated whether *P. mascittii* functions as a vector of *Leishmania* species.

Although it is impossible to prove where and how our patient acquired the *L. infantum* parasites, the most likely explanation is an infection in a novel autochthonous focus in Germany or The Netherlands. We believe that physicians in and outside Europe should be alert to the possibility of leishmaniasis in patients presenting with fever of unknown origin, hepatosplenomegaly, and pancytopenia, even if there is no history of traveling to classic endemic areas.

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