Occurrence of *Leishmania infantum* Parasitemia in Asymptomatic Blood Donors Living in an Area of Endemcity in Southern France

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Visceral leishmaniosis (VL) due to *Leishmania infantum* (*L. chagasi*) is a lethal disease if untreated, but asymptomatic *L. infantum* infections have been reported previously. A better understanding of parasite transmission, dissemination, and survival in the human host is needed. The purpose of this study was to assess whether *L. infantum* circulated in peripheral blood of subjects with no history of VL. Sera from 565 blood donors were screened by Western blotting to detect *Leishmania*-specific antibodies and identify individuals with probable past exposure to *Leishmania*. Seropositivity was found in 76 donors whoseuffy coats were examined by PCR and direct culture. The parasite minicircle k inetoplast DNA was amplified from blood samples of nine donors. Promastigotes were detected by culture in blood samples from nine donors. Only two donors were PCR and culture positive. These results indicate that *L. infantum* circulates intermittently and at low density in the blood of healthy seropositive individuals, who thus appear to be asymptomatic carriers. Implications for the safety of blood transfusion are discussed.

The visceral form of leishmaniosis (VL) affects approximately half a million new patients each year. Due to systemic parasite dissemination, the disease is fatal if untreated (27). The main areas of concern are Sudan, Eastern India, Bangladesh, and Nepal ( *Leishmania donovani*) and Brazil and the area around the Mediterranean ( *Leishmania infantum*) (34).

Those infected with the visceralotropic *Leishmania* species may, however, remain asymptomatic (3, 27). The documentation of individuals who have no history of VL but whose leishmanin skin tests (LST) are positive—specific evidence of delayed antileishmanial hypersensitivity—is not new (3, 21, 26). The mechanisms implicated in susceptibility in humans are not fully elucidated, although much has been learned about leishmaniosis in a murine model (29). Immunosuppression, such as in AIDS patients, is one of the factors responsible for increased vulnerability to a primary *Leishmania* infection or to reactivation of a latent infection (14; for a review, see reference 1).

Coinfection with human immunodeficiency virus (HIV) and *Leishmania* is becoming increasingly frequent; to date, 1,400 VL-AIDS cases have been reported in southern Europe (34).

The documentation of occult *Leishmania* in healthy subjects is important so that researchers can obtain more knowledge about parasite reservoir and transmission and a better understanding of the pathways of parasite dissemination and its capacity for surviving in the host. Parasite circulation in peripheral blood has been reported in asymptomatic *Leishmania donovani* and *Leishmania tropica* infections (6, 9, 31) and in cured and inapparent *Leishmania braziliensis* infection (10, 11, 19), but except for our preliminary report (15), no documentation of parasitemia in healthy *L. infantum*-seropositive individuals exists.

We showed previously (21) that the positivity of the LST correlated with the presence of specific antileishmanial antibodies evidenced by Western blotting as typical 14- and/or 18-kDa bands. More recently, the detection of these antibodies before VL diagnosis proved effective in distinguishing cases of VL following primary *Leishmania* infection from cases originating from the reactivation of a latent infection in HIV-positive patients (14). In this study, the 14- and/or 18-kDa bands revealed by Western blotting with sera of blood donors were used to identify individuals who were probably exposed to *Leishmania*. Our aim was to assess whether *L. infantum* parasitemia occurred in asymptomatic *Leishmania*-seropositive subjects with no history of VL.

From the standpoint of public health, demonstration of the parasite circulation in the blood must be followed by documentation of its transmission by blood transfusion, which has not yet been provided. Decisions regarding the implementation of the *Leishmania* screening test and the safety of blood transfusions are discussed in this context.

**MATERIALS AND METHODS**

**Subjects and experimental protocol.** Blood obtained from the Monaco Blood Bank was from donors living in neighboring areas where *L. infantum* is endemic (20) and having no history of VL. Blood from 565 donations was screened over a period of 12 months (April 1996 to March 1997). On the day of donation, sera were analyzed by Western blotting, and theuffy coats corresponding to the sera which revealed the typical 14- and/or 18-kDa leishmanial bands (21, 33) were further examined. Three aliquots (3 ml each) of the 1-day-old (stored at 4°C)uffy coats were stored at ~20°C in EDTA containing vacutainer tubes (Becton Dickinson, Meylan, France) until DNA extraction and amplification by PCR. The buffy coats or peripheral blood mononuclear cells (PBMC) were seeded for culture.

**Control sera.** Presumably negative control sera were obtained from 141 individuals living in areas free of *L. infantum*; these individuals were blood donors (*n* = 50, Bourg-en-Bresse, France) or women who were seronegative for toxoplasmosis during their pregnancies (Parasitology Laboratories, University Hosp...
pitals of Reims [n = 50] and Angers [n = 41, France]. The sera of patients cured of VL were used as positive controls.

**Guidelines for human research.** Informed, written consent from all participants and the approval of the local ethics committees at our institutions were obtained for this study.

**Isolation of parasites by culture.** Three to six milliliters of buffy coats from *Leishmania*-seropositive subjects was cultured in 25 ml of RPMI 1640 complete medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin [100 U/ml], streptomycin [100 µg/ml], and 10% heat-inactivated fetal calf serum). Alternatively, PBMC were isolated by centrifugation of buffy coats (diluted 1:1 in 0.9% NaCl) over lymphocyte separation medium (Eurobio, Les Ulis, France). The interface cells were washed, and 5 x 10^6 to 15 x 10^6 PBMC were seeded at 1 x 10^6 to 3 x 10^6 cells per ml in RPMI 1640 complete medium or in Schneider’s medium as described in reference 17. All cultures were maintained at 25°C for 6 months (or until positive) and were inspected by inverted contrast phase microscopy twice per month. The medium was changed twice per month for 2 months and then once per month. We verified that after approximately 1 month most human cells were taking up trypan blue.

**Serological screening.** The bands at 14 and/or 18 kDa, indicative of a previous asymptomatic infection with *L. infantum* (14, 21), were revealed by the sera from 76 donors (13.4%) to Monaco Blood Bank. Antibodies detecting both bands were found in 25 serum samples, and antibodies detecting one band at 14 or 18 kDa were found in 20 or 31 serum samples, respectively. Figure 1 shows the characteristic Western blot profiles obtained with the sera of 16 donors found to be *Leishmania* seronegative and an aliquot of distilled water) were included in each PCR run to detect contamination which could lead to false-positive results. The amplification products were visualized after electrophoresis on a 3% agarose gel containing ethidium bromide.

**RESULTS**

**Serological screening.** The bands at 14 and/or 18 kDa, indicative of a previous asymptomatic infection with *L. infantum* (14, 21), were revealed by the sera from 76 donors (13.4%) to Monaco Blood Bank. Antibodies detecting both bands were found in 25 serum samples, and antibodies detecting one band at 14 or 18 kDa were found in 20 or 31 serum samples, respectively. Figure 1 shows the characteristic Western blot profiles obtained with the sera of 16 donors found to be *Leishmania* seronegative and an aliquot of distilled water) were included in each PCR run to detect contamination which could lead to false-positive results. The amplification products were visualized after electrophoresis on a 3% agarose gel containing ethidium bromide.
to the same (HIV positive) VL patient were found to be analyzed by Western blotting; two persons who donated blood that 6 of the former and 2 of the latter had had a blood patients) or not infected with HIV (18 patients), and found examined the files of 50 VL patients, infected with HIV (32 positive samples were culture negative (Fig. 1).

Amplification of parasite kDNA in buffy coats of Leishmania-seropositive blood donors. Buffy coats from 73 Leishmania-seropositive donors were analyzed, since buffy coats from 3 Leishmania-seropositive donors found to be hepatitis C virus seropositive were discarded from the study. Under the experimental conditions described in Materials and Methods, the Leishmania kDNA was detected in the buffy coats of nine Leishmania-seropositive blood donors with primers RV1 and RV2. Primers 13A and 13B were found to be less efficient and amplified parasite kDNA in seven of these nine positive samples. Figure 2 shows examples of amplifications with primers RV1 and RV2 (Fig. 2A and B) and primers 13A and 13B (Fig. 2C) for four PCR-positive and three PCR-negative donors. The blood of five PCR-positive donors was drawn during the period when Leishmania-transmitting sandflies (May to October) are active, while the remaining PCR-positive samples were obtained from blood donated outside this period (Fig. 1).

Detection of parasites by culture. Leishmania infantum promastigotes were detected in cultures of blood cells from 9 donors. These cultures were not carried out under standardized conditions (see Materials and Methods). Six positive samples were obtained from cultures of 6 ml of buffy coats (corresponding to approximately 60 ml of blood), and the remaining three positive samples were PBMC cultures (corresponding to approximately 10 ml of blood). In all cases a long incubation period (1 to 6 months) was necessary to detect parasites. All isolated strains were typed as Zymodeme MON-1. For one donor the culture was positive in two independent blood donations (a culture of the buffy coat in April and a culture of PBMC in October). For this donor the Leishmania kDNA was also detected by PCR. Evidence of the parasitemia in two PCR-positive donors was also provided by culture. The remaining PCR-positive samples were culture negative (Fig. 1).

Retroactive study of files and sera of VL patients. We examined the files of 50 VL patients, infected with HIV (32 patients) or not infected with HIV (18 patients), and found that 6 of the former and 2 of the latter had had a blood transfusion. Sera from 10 persons who donated blood to two patients that received transfusions (six donors for one patient and four donors for the second patient) were recovered and analyzed by Western blotting; two persons who donated blood to the same (HIV positive) VL patient were found to be Leishmania seropositive. Analyses of sera drawn previously from the recipient showed that this patient was Leishmania seropositive before the transfusion.

**DISCUSSION**

In this study we screened blood from the Monaco Blood Bank obtained from 565 donors living in an area where *L. infantum* is endemic. The Leishmania-specific antibodies were revealed by Western blotting in the sera of 76 donors which showed the characteristic 14- and/or 18-kDa bands. The presence of the parasite was evident in the blood of 16 Leishmania-seropositive donors either by parasite kDNA amplification or by direct culture.

The knowledge that some individuals with no history of VL due to *L. infantum* present a positive LST (showing past exposure to the parasite) is not new (3, 21, 26). We showed previously (21) that the positivity of LST was in biological concordance (80%) with the presence of antibodies directed against 14- and/or 18-kDa fractions of *L. infantum*. In this study, six control serum samples of persons living in regions free of leishmaniosis (of 141 samples tested) also detected the typical 14- and/or 18-kDa bands, but five of these six samples should be disregarded, since previous travel to regions where *Leishmania* is endemic was mentioned by donors in interviews. Although false-positive and false-negative results cannot be excluded when one serological test is used, and since the aim of the study was qualitative, rather than quantitative, we selected the buffy coats of *Leishmania*-seropositive donors to search for the parasite.

Parasite kDNA was amplified in blood samples from nine donors. The live *L. infantum* promastigotes were detected by culture in blood samples from nine donors. Only two donors were PCR positive and culture positive. A second blood donation from the culture-positive donors was obtained several months after the first; only one of these samples was culture positive, and all nine samples were PCR negative. These results indicate that the density of *Leishmania* in the peripheral blood is low and that parasitemia is probably episodic. Why PCR, which is a powerful technique, gives a negative result for a blood sample from a culture-positive donor is puzzling. However, amplification was performed on DNA extracted from approximately 30 ml of blood, so even if minicircle kDNA amplification may, theoretically, detect 1 parasite in 1 million cells (28), our findings indicate that not every 30 ml of blood carried an amastigote kDNA target sequence. The direct culture was performed on the equivalent of 10 to 60 ml of blood, a poor way to amplify *Leishmania*, and we were unable to determine the optimal culture conditions. In all cases the detection of parasites was made possible by the maintenance of the cultures for unusually long periods. Several nonexclusive
We estimate that prior to any kind of preparation of packed erythrocytes, a blood unit contains $2 \times 10^{10}$ to $3 \times 10^{10}$ leukocytes. After the first step of preparation, i.e., the removal of theuffy coat after centrifugation, the blood unit contains roughly $5 \times 10^{10}$ leukocytes. After the second step, i.e., deleukocytation by filtration, the unit contains fewer than $10^9$ leukocytes. Therefore, assuming that parasite transmission is proportional to leukocyte concentration, a risk score of 1 assigned to an initial, undeleukocyted unit would decrease to $10^{-3}$ after deleukocytation. At present, all blood products in Morocco (as of 1 April 1998) are deleukocyted. Blood is not conserved prior to deleukocytation, preventing the possible release of intracellular agents by cell lysis during storage. Finally, it should be emphasized that screening blood for one pathogenic agent protects against this agent alone, while the deleukocytation of blood products eliminates several pathogens (e.g., cytomegalovirus, but also as-yet- unidentified agents), prevents alloimmunization, and improves the well-being of the blood recipient.

In conclusion, we believe that considering present constraints of time, money, and knowledge, researchers should invest first in the preparation of blood products and then in donor testing to protect recipients of blood transfusions against \textit{L. infantum}.

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REFERENCES


